Universidad Autónoma de Madrid
Facultad de Ciencias
Departamento de Biología Molecular
Centro de Biología Molecular Severo Ochoa

# El Ribosoma de *Leishmania* y su interacción con el sistema inmunológico del hospedador.

Memoria Presentada por:
Laura Ramírez García. Licenciada en Biología
Para optar al grado de Doctor en Biología Molecular por la Universidad Autónoma de Madrid

Tesis dirigida por: Manuel Soto Álvarez

FACULTAD DE CIENCIAS
UNIVERSIDAD AUTÓNOMA DE MADRID

**DEPARTAMENTO DE BIOLOGÍA MOLECULAR**

MANUEL SOTO ÁLVAREZ, PROFESOR TITULAR DEL DEPARTAMENTO DE BIOLOGÍA MOLECULAR DE LA FACULTAD DE CIENCIAS DE LA UNIVERSIDAD AUTÓNOMA DE MADRID, CERTIFICA:

que he dirigido la Tesis Doctoral titulada "El ribosoma de *Leishmania* y su interacción con el sistema inmunológico del hospedador" realizada por Dña. Laura Ramírez García.

Y para que así conste firmo este certificado en Madrid, 22 mayo de 2014

Dr. Manuel Soto
Profesor Titular Dpto. Biología Molecular.
Centro de Biología Molecular Severo Ochoa
Facultad de Ciencias
Universidad Autónoma de Madrid
28049-Madrid (España)
Teléfono: +34 91 1964471/ +34 91 4978045
Fax: +34 91 1964420
E-mail: manuel.soto@uam.es/
msoto@cbm.csic.es

FACULTAD DE CIENCIAS
UNIVERSIDAD AUTÓNOMA DE MADRID

**DEPARTAMENTO DE BIOLOGÍA MOLECULAR**

**D. Miguel Ángel Íñiguez Peña, con DNI 50164108G, en calidad de Coordinador del Doctorado en Biociencias Moleculares de la Universidad Autónoma de Madrid,**

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Su aprobación a la solicitud de que la presentación de la tesis doctoral de Dña. Laura Ramírez García y dirigida por el Dr. Manuel Soto Álvarez, titulada "El ribosoma de *Leishmania* y su interacción con el sistema inmunológico del hospedador" se realice como compendio de publicaciones según la normativa vigente sobre el procedimiento relativo al tribunal, defensa y evaluación de la tesis doctoral en la UAM.

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Y para que conste a todos los efectos,

*firmo en Madrid, a 11 de marzo de 2014*

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*Coordinador del Doctorado en Biociencias Moleculares*
*Departamento de Biología Molecular, Facultad de Ciencias*
*Universidad Autónoma de Madrid*

Faculta de Ciencias
Universidad Autónoma de Madrid
Ciudad Universitaria de Cantoblanco
28049 Madrid

A mis padres

# AGRADECIMIENTOS

谢谢

Merci

Dank

благодаря

ধন্যবাদ

Thanks

OBRIGADA

شكرا

感謝

Спасибо

ευχαριστώ

ขอบคุณ

Takk

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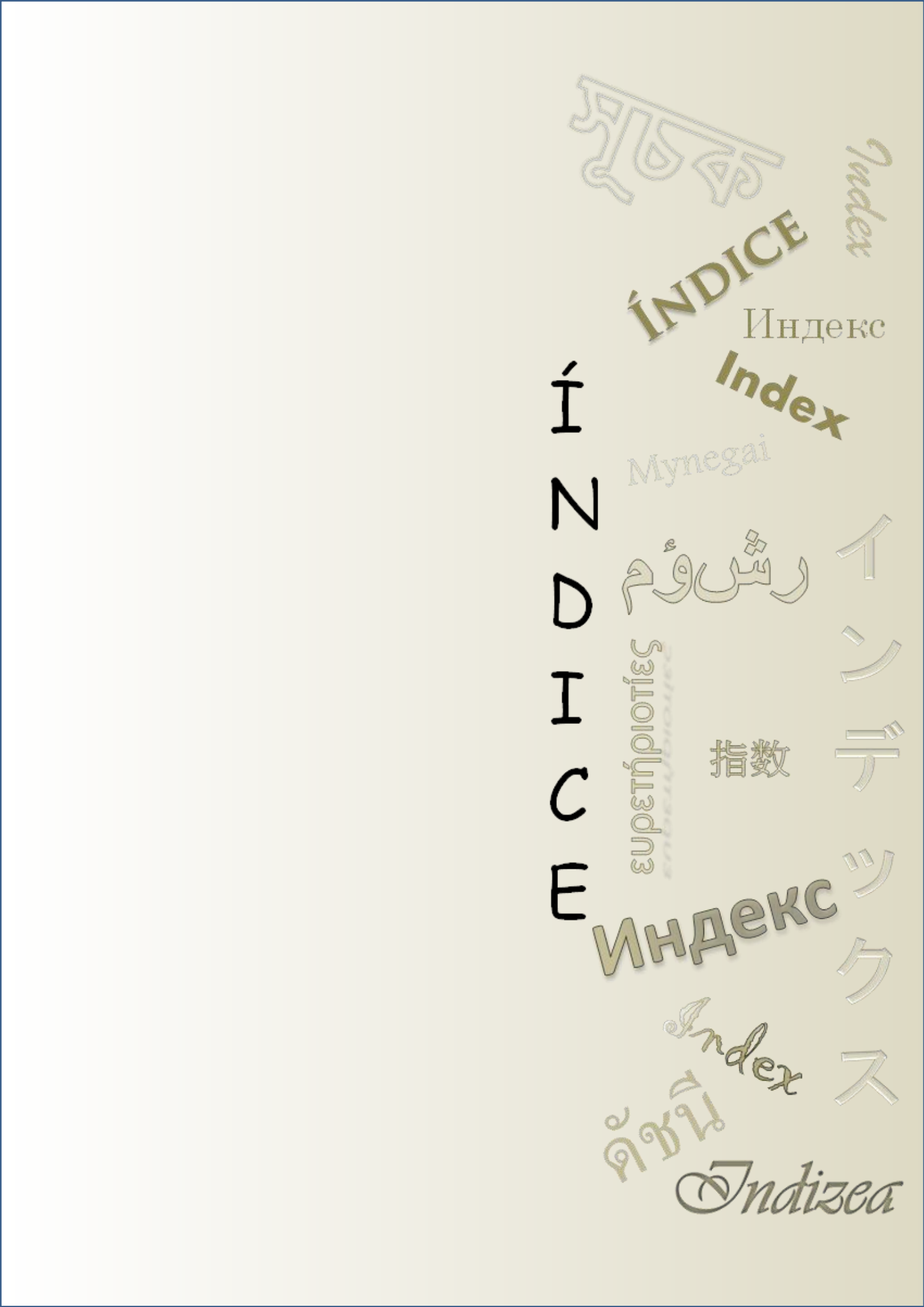
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# ABREVIATURAS

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Abbreviations

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略語

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Abbreviazioni

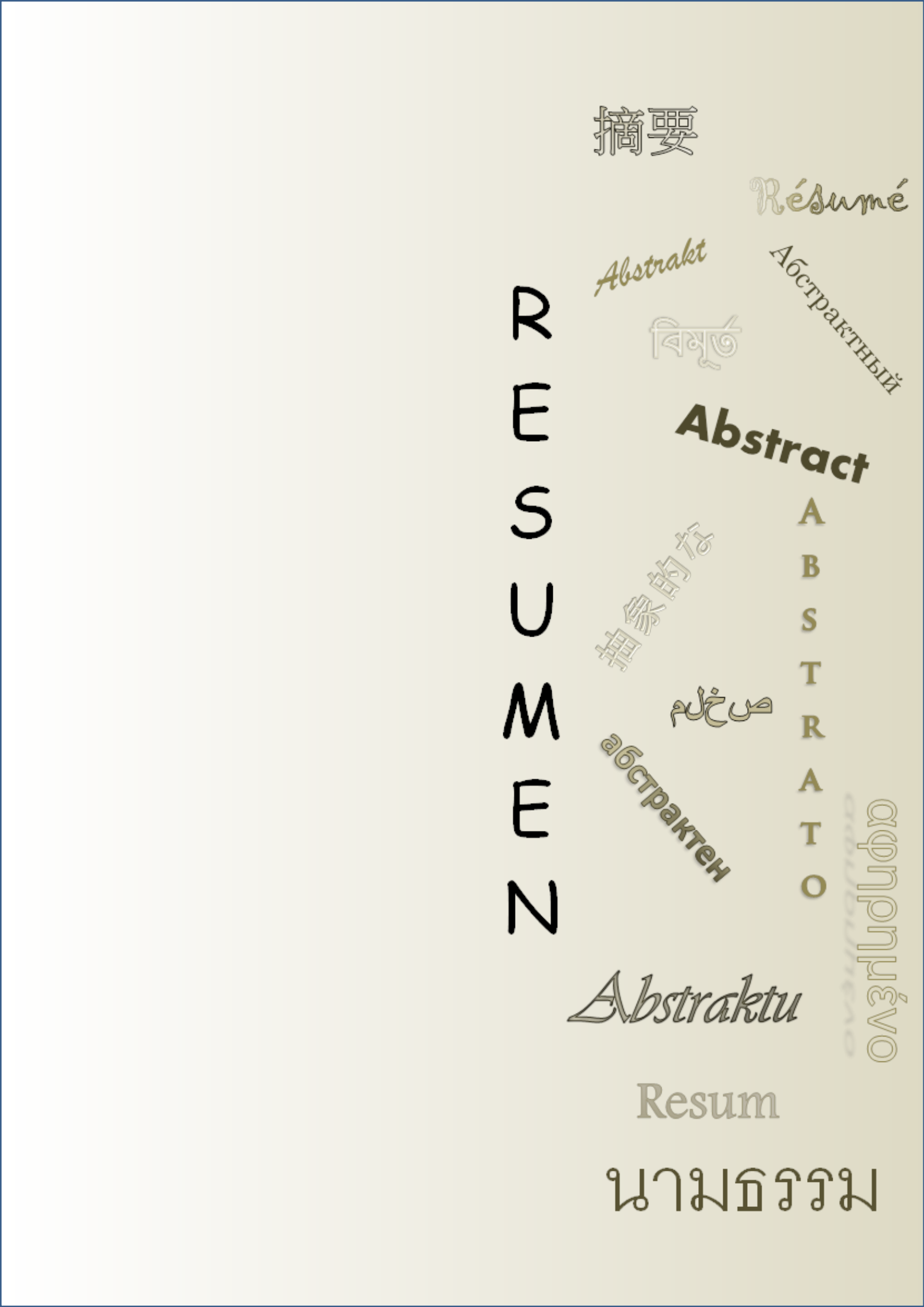
abreviaturas

Συντομογραφίες

Laburdurak

| | Español | Inglés |
|---|---|---|
| • **BCG:** | Bacilo de Calmette-Guerin | Bacillus Calmette–Guérin |
| • **CD:** | Marcadores de diferenciación | Clusters of Diferentiation |
| • **CR:** | Receptor del Complemento | Complement Receptor |
| • **O.D.:** | Densidad Óptica | Optical Density |
| • **DDC:** | Células Dendríticas Dérmicas | Dermal Dendritic Cells |
| • **DNL:** | Ganglios Linfáticos Drenantes | Drenant Limph Nodes |
| • **DTH:** | Hipersensibilidad retardada | Delayed-Type Hypersensitivity |
| • **GP63:** | Glicoproteína de 63 kDa | Surface Glycoprotein of 63 kDa |
| • **HSP:** | Proteína de Choque Térmico | Heat Shock Protein |
| • **KMP11:** | Proteína de membrana de 11 kDa del orden *Kinetoplastida*. | Kinetoplastid Membrane Protein 11 |
| • **LACK:** | Homólogo del receptor para la proteína kinasa C activada en *Leishmania* | *Leishmania* homolog of receptors for Activated C-Kinase |
| • **Lc:** | *Leishmania chagasi* | *Leishmania chagasi* |
| • **LC/CL:** | Leishmaniosis cutánea | Cutaneous leishmaniasis |
| • **LCD:** | Leishmaniosis cutánea difusa | Diffuse cutaneous leishmaniasis |
| • **LDPK:** | Leishmaniosis dérmica post *kala-azar* | Post-kala-azar dermal leishmaniasis |
| • **Lm:** | *Leishmania major* | *Leishmania major* |
| • **LMC:** | Leishmaniosis mucocutánea | Mucocutaneous leishmaniasis |
| • **LPG:** | Lipofosfoglicano | Lipophosphoglycan |
| • **LRP:** | Extractos de ribosomas de *Leishmania* | *Leishmania* ribosomal proteins |
| • **LV/VL:** | Leishmaniosis visceral | Visceral leishmaniasis |
| • **LVC/CVL:** | Leishmaniosis visceral canina | Canine visceral leismaniasis |
| • **MBL:** | Lectina de unión a manosa | Mannose-binding lectin |
| • **MRP:** | Proteínas ribosómicas de ratón | Mouse Ribosomal Proteins |
| • **NETs:** | Trampas extracelulares de neutrófilos | Neutrophil Extracellular Traps |
| • **NK:** | Células asesinas natas | Natural Killer |
| • **ODN:** | Oligodesoxirribonucleótido | Oligodeoxynucleotides |

| | | |
|---|---|---|
| • **OMS/WHO:** | Organización Mundial de la Salud | World Health Organization |
| • **OPD:** | 1,2-Fenilendiamina | 1,2-Phenylenediamine |
| • **PRR** | Receptores de Reconocimiento de Patógenos | Patogen Recognition Receptor |
| • **RT:** | Temperatura Ambiente | Room Temperature |
| • **SLA:** | Antígeno Soluble de *Leishmania* | Soluble *Leishmania* Antigen |
| • **Tcm:** | Células T memoria centrales | T cell central memory |
| • **TCR:** | Receptor de células T | T cell receptor |
| • **Tem:** | Células T memoria efectoras | T cell efector memory |
| • **TGF:** | Factor de crecimiento transformante | Transforming Growth Factor |
| • **Th:** | Linfocito T cooperador | T helper |
| • **TLR:** | Receptor de tipo Toll | Toll-like receptor |
| • **TNF:** | Factor de Necrosis Tumoral | Tumor Necrosis Factor |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La leishmaniosis es una zoonosis provocada por protozoos del género *Leishmania*. Hoy en día todavía no existe una vacuna disponible para su uso en seres humanos. En este trabajo se ha estudiado el posible uso de los ribosomas como herramientas inmuno-profilácticas frente a las infecciones por *Leishmania.*

Primero, se analizó la antigenicidad de las proteínas ribosómicas de *Leishmania* (LRP) usando muestras obtenidas a partir de perros infectados de forma natural que mostraban diferentes síntomas clínicos, desde animales asintomáticos hasta sintomáticos. Los resultados demostraron que la mayoría de los perros infectados tienen anticuerpos circulantes contra los ribosomas y que la mayoría de las proteínas que constituyen el ribosoma son antigénicas. En este sentido, el LRP ha demostrado ser mejor candidato para el diagnóstico de la leishmaniosis canina que el Antígeno Soluble de *Leishmania* (SLA), ya que los ensayos de ELISA basados en LRP presentan valores con mayor sensibilidad y especificidad que los que emplean SLA.

En segundo lugar, este trabajo se centró en el estudio de la capacidad de las partículas ribosómicas, así como alguno de sus componentes, de generar protección frente a infecciones producidas por varias especies de *Leishmania.* Usando diferentes modelos de infección basados en el empleo de ratones BALB/c se observó que la vacunación combinada de LRP junto con un adjuvante estimulador de respuestas celulares era capaz de producir respuestas inmunes protectoras contra el desarrollo de leishmaniosis cutáneas y viscerales (causadas por la infección con *Leishmania major* y *Leishmania amazonensis* o *Leishmania chagasi*, respectivamente). Además, los ratones protegidos frente al desarrollo de leishmaniosis cutánea (LC) causada por *L. major* por su vacunación con LRP + CpG-ODN (oligodeoxirribonucleótidos con motivos CpG no metilados), fueron capaces de resistir una segunda infección. Estos efectos protectores se deben principalmente a la inducción de respuestas mediadas por IFN-γ acompañadas con el control de la producción de las citoquinas IL-10 e IL-4 en respuesta a antígenos del parásito. Por otra parte, se caracterizaron y clonaron cuatro de las proteínas que forman parte del ribosoma. Sus versiones recombinantes LmS4, LmS6, LmL5 y LmL3 demostraron ser reconocidas por el suero tanto de pacientes humanos infectados como caninos. Entre ellas LmL3 y LmL5 fueron capaces de generar una mayor respuesta protectora cuando fueron ensayadas como vacunas en presencia de CpG-ODN, en el modelo BALB/c-*L. major*. Por este motivo, el análisis de su potencial profiláctico se extendió a otros modelos de infección empleando la misma cepa de ratones y tres

especies diferentes de parásitos *Leishmania braziliensis, L. amazonensis* y *L. chagasi.* En todos los casos se observó una respuesta protectora asociada a la administración de las vacunas basadas en LmL3, LmL5 y CpG-ODN. Los ratones vacunados mostraron cargas parasitarias más bajas en los órganos infectados, así como una reducción de las lesiones cutáneas (cuando se infectan con las especies cutáneas *L. braziliensis* y *L. amazonensis*) en comparación con los grupos control inmunizados con el adyuvante o diluyente de la vacuna. La protección se relacionó principalmente con la producción específica de IFN-γ por parte de LmL3 y LmL5 (mayor en magnitud para la proteína LmL5) y el control de la respuesta específica de IL -10 inducida por la LmL3 en los ratones infectados no vacunados.

En conjunto, los resultados aquí descritos indican que los constituyentes del ribosoma de *Leishmania* pueden ser un componente relevante de una vacuna profiláctica contra la leishmaniosis humana.

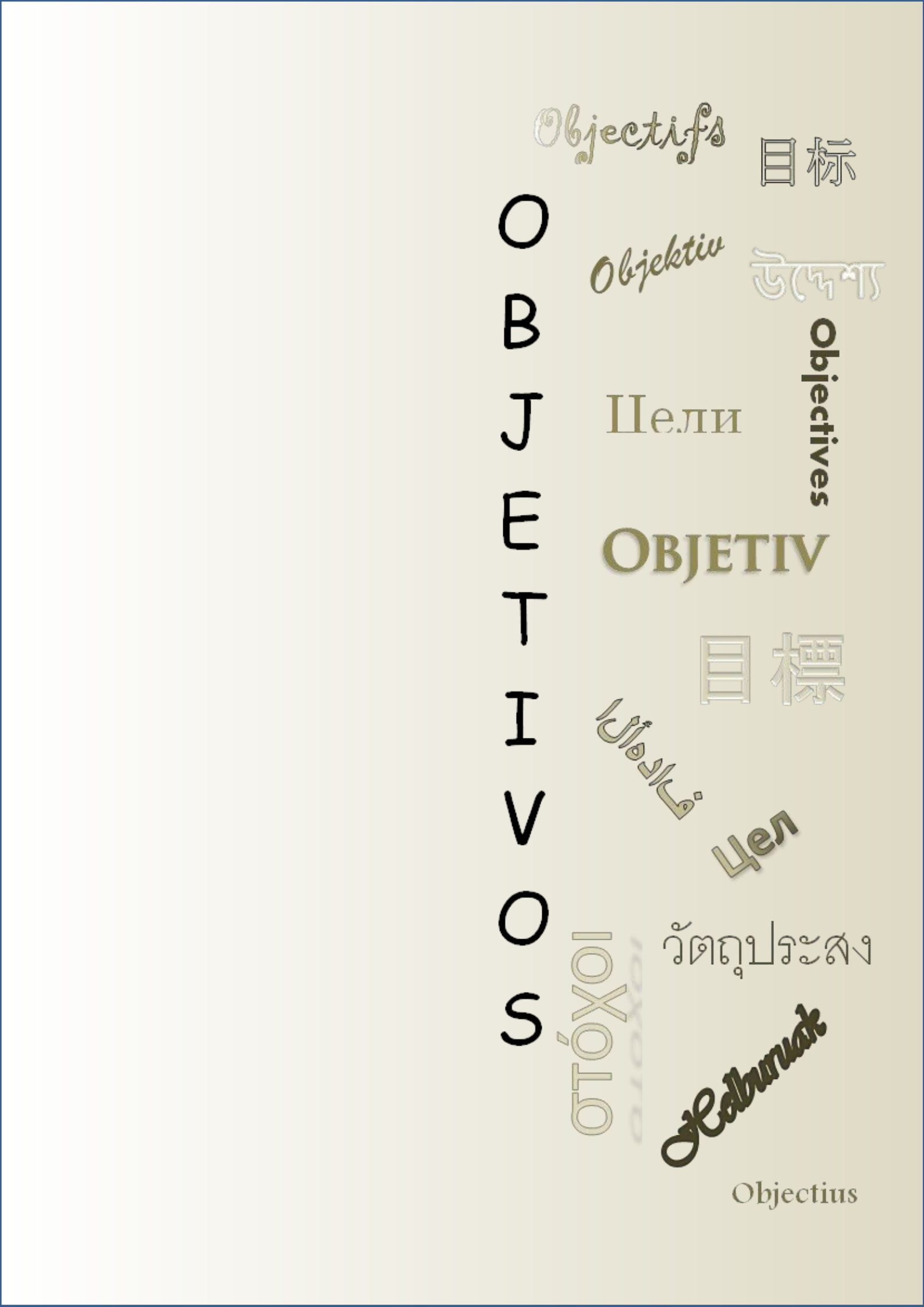
Leishmaniasis is a zoonotic disease caused by protozoa of the genus *Leishmania*. Nowadays there is still no vaccine available for use in humans. In this work, the possible use of ribosomes as immuno-prophylactic tools against the development of disease after infection with *Leishmania* has been studied.

First, we have analyzed the antigenicity of the *Leishmania* ribosomal proteins (LRP) using samples obtained from naturally infected dogs showing different clinical symptoms ranging from asymptomatic to symptomatic forms. The results demonstrated that most of the infected animals possess circulating antibodies against ribosomes and that most of the ribosomal protein constituents are antigenic. In this sense, the LRP have been proved to be a better candidate than Soluble *Leishmania* Antigen (SLA) for diagnosis of canine leishmaniasis, since ELISA assays based on LRP show higher sensitivity and specificity values than SLA based assays.

Second, this work has focused on the study of the ability of the ribosome particles, and some of its components, to generate protection against infections caused by several species of *Leishmania*. Using different models of infection based on the BALB/c mice, it was observed that vaccination with the combination of LRP and adjuvants that stimulate cellular responses was able to induce immune responses that protects against the development of dermal and visceral leishmaniasis (caused by infection with *L. major* and *L. amazonensis* or *L. chagasi*, respectively). Moreover, mice protected against cutaneous leishmaniasis due to *L. major* infection by vaccination with LRP + CpG-ODN were able to resist a secondary infection. These protective effects are mainly due to the induction of IFN-γ mediated responses accompanied by the control of production of IL-10 and IL-4 cytokines in response to parasite antigens. In addition, four ribosomal antigenic proteins have been characterized and cloned. Their recombinant versions, LmS4, LmS6, LmL3, LmL5, were recognized by sera from *Leishmania* infected humans and dogs. Among them, LmL3 and LmL5 were able to generate a stronger protective response when tested as vaccines in the *L. major*-BALB/c model in the presence of CpG-ODN. For this reason the analysis of their prophylactic properties was extended to other models of infection using the same mice strain and three different parasites species: *L. braziliensis*, *L. amazonensis* and *L. chagasi*. In all tested models a protective response of the LmL3, LmL5 and CpG-ODN based vaccine was observed. Vaccinated mice showed lower parasite burdens in the infected organs as well as reduced cutaneous lesions (when infected with the cutaneotropic species *L. braziliensis* and *L. amazonensis*) when compared with control groups immunized with the vaccine adjuvant or diluent. The immune correlate to protection was mainly related with the LmL3 and LmL5 specific IFN-γ production (higher in magnitude for the LmL5 protein) and

control of the LmL3-induced IL-10 specific response observed in non-vaccinated infected mice.

Altogether, results here depicted indicate that *Leishmania* ribosomal constituents may be relevant components of a protective prophylactic vaccine against human leishmaniasis.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En el laboratorio donde se ha desarrollado este trabajo se había demostrado previamente que la inmunización de ratones con proteínas del ribosoma en presencia de CpG-ODN generaba un estado de inmunidad frente a la infección por *L. major*, tanto en ratones susceptibles como en ratones resistentes.

En este trabajo se decidió ampliar el conocimiento de las interacciones de las proteínas ribosómicas con el sistema inmunológico del hospedador vertebrado en infecciones naturales así como completar el análisis del potencial profiláctico de estos extractos, empleando modelos de leishmaniosis experimentales en el ratón. Por otra parte, se quiso de caracterizar algunas de las proteínas protectoras del ribosoma para desarrollar una vacuna definida basada en sus versiones recombinantes. Con estas hipótesis se plantean los siguientes objetivos específicos:

- Análisis de la antigenicidad de los extractos de ribosomas de *Leishmania* en leishmaniosis caninas naturales.
- Análisis de la capacidad de los ratones BALB/c vacunados con LRP e infectados con *L. major*, de resistir a una segunda infección.
- Estudio del efecto de la vacunación con LRP empleando modelos de infección experimental en el ratón con otras especies de *Leishmania*: *L. chagasi* y *L. amazonensis.*
- Caracterización de cuatro proteínas antigénicas del ribosoma y obtención de sus versiones recombinantes (LmS4, LmS6, LmL3 y LmL5).
- Análisis de la inmunogenicidad y la protección generada por las proteínas candidatas en el modelo *L. major*-ratón BALB/c.
- Estudios de protección en otros modelos de LC en el ratón: *L. braziliensis* y *L. amazonensis*, y un modelo de LV (leishmaniosis visceral): *L. chagasi*.

# INTRODUCCIÓN

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INTRODUÇÃ

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Introduzione

## *Leishmania.*

*Leishmania* es un protozoo parásito con un ciclo de vida digenético que pertenece al orden *Kinetoplastida*. Se conocen unas 30 especies de *Leishmania* de las cuales 20 son patógenas (Ashford 2000). Su infección en los hospedadores vertebrados provoca un conjunto de enfermedades denominadas leishmaniosis. La Organización Mundial de la Salud (OMS) considera que las leishmaniosis son endémicas en 98 países localizados en las regiones tropicales y subtropicales del planeta. Se calcula que unos 14 millones de personas se encuentran afectadas por la enfermedad y se estima que la cifra aumenta en unos 1,6 millones al año. El número de personas en riesgo de contraer la enfermedad, al residir o visitar regiones endémicas es aproximadamente de 350 millones (http://www.who.int/leishmaniasis/en).

En el hospedador invertebrado, la mosca de la arena, el parásito se encuentra en forma promastigote. El promastigote tiene vida extracelular, posee flagelo y tiene forma alargada. En el hospedador vertebrado (generalmente mamíferos), el parásito se replica en el interior de las células del sistema mononuclear fagocítico en forma amastigote. Esta forma carece de flagelo y tiene morfología redondeada (Banuls, Hide et al. 2007). El ciclo de transmisión del patógeno comienza cuando la mosca hembra se alimenta de la sangre de un hospedador infectado (Fig. 1). Junto con la sangre, ingiere macrófagos que contienen las formas amastigotes. En su tubo digestivo se diferencian en promastigotes procíclicos, desarrollando el flagelo y multiplicándose activamente por fisión binaria. Más adelante, estos promastigotes sufren cambios en los motivos de glicosilación de sus moléculas de superficie, tanto glicoproteínas como un glicolípido muy abundante en la forma promastigote conocido como lipofosfoglicano (LPG), que provocan su transformación en las formas metacíclicas (altamente infectivas) y su migración hacia la probóscide de la mosca (Sacks 2001). Estas formas son inoculadas al hospedador vertebrado durante la picadura (Pimenta, Turco et al. 1992). En éste, los promastigotes infectan macrófagos y en el interior de la vacuola parasitófora (de origen lisosómico) se diferencian en amastigotes debido a las condiciones de temperatura y acidez (Rittig and Bogdan 2000). Los amastigotes son formas replicativas que sufren sucesivas divisiones binarias aumentando su número e infectando a nuevos macrófagos, lo que contribuye a su diseminación por el sistema vascular (Chang and Fong 1983).

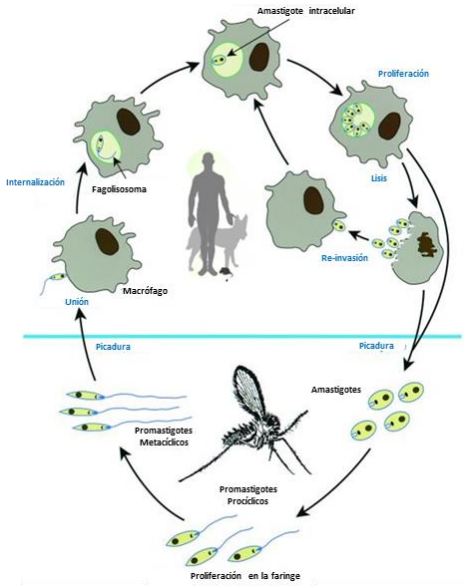


**Figura 1: Ciclo de vida del parásito *Leishmania*.** Modificado de (Handman 2001).

Los parásitos son transmitidos por diferentes vectores. En el viejo mundo, el género transmisor es *Phlebotomus*, distribuido en países de África (norte y región tropical), Asia (sudoeste y región central), Oriente Medio y el sur de Europa. El género *Lutzomyia* es responsable de la transmisión de la enfermedad en países del nuevo mundo: Centroamérica y Sudamérica. En el hombre aparecen diferentes formas de leishmaniosis (Herwaldt 1999). Dependiendo de sus síntomas clínicos, se clasifican como:

**Leishmaniosis cutánea** (**LC**): es la forma más común (50-75% de los nuevos casos) y menos grave de leishmaniosis. Se caracteriza por la formación de lesiones ulcerativas en la zona donde ocurrió la picadura. En ocasiones se cura de forma espontánea, pero queda una cicatriz más pigmentada que el resto de la piel. Existe una variante de leishmaniosis cutánea, conocida como **leishmaniosis cutánea difusa (LCD)**, consistente en lesiones cutáneas no ulcerativas diseminadas por zonas extensas del cuerpo y en la zona facial.

**Leishmaniosis mucocutánea (LMC):** aparece fundamentalmente en pacientes que se recuperaron de la LC provocada por la infección de algunas especies del parásito. Es una forma de leishmaniosis con baja tasa de mortalidad pero que puede generar lesiones desfigurantes en distintas áreas mucosas: mucosa nasal, faringe, laringe, tráquea y paladar.

**Leishmaniosis visceral o kala-azar** (**LV**): es la forma más grave de la enfermedad y provoca la muerte del paciente si éste no es tratado. El parásito se disemina por los órganos internos causando episodios de fiebre, pérdida de peso, anemia e hinchazón del bazo y del hígado. En algunas ocasiones, en pacientes tratados

con éxito de LV, aparece un tipo de leishmaniosis cutánea denominada **leishmaniosis dérmica post-kala-azar (PKDL)**. Se caracteriza por la aparición de múltiples infiltrados nodulares, especialmente en la cara. Los casos de PKDL tienen gran importancia epidemiológica, ya que los pacientes actúan como reservorio de parásitos (Das, Biswas et al. 2009).

Los síntomas clínicos de las diferentes leishmaniosis dependen de tres factores. En primer lugar, de la especie de *Leishmania* infectante. En la Tabla 1 se incluyen las especies más representativas causantes de las diferentes formas de la enfermedad.

| **TROPISMO** | **ESPECIES** | |
|---|---|---|
| | **NUEVO MUNDO** | **VIEJO MUNDO** |
| **Visceral** | *L. infantum* (=*L. chagasi*). | *L. donovani, L. infantum.* |
| **Dérmico** | *L. amazonensis, L. braziliensis, L. mexicana, L. panamensis, L. guayanensis, L. peruviana.* | *L. major, L. tropica, L. donovani, L. infantum, L. aethiopica.* |
| **Mucocutáneo** | *L. braziliensis, L. panamensis.* | |

**Tabla 1:** Especies de *Leishmania* y su tropismo en el hospedador vertebrado.

Cada una de las especies de *Leishmania* genera un tipo de patología aunque, en algunos casos, especies que normalmente producen LC como *L. amazonensis* pueden visceralizar (Barral, Pedral-Sampaio et al. 1991). Esta complicación está relacionada con el segundo factor implicado en la relación parásito-hospedador, ya que el estado del sistema inmunológico del hospedador así como sus condiciones fisiológicas pueden influir en el desarrollo de la patología. La malnutrición y el estrés se consideran favorecedores de la enfermedad y, de hecho, las epidemias de LV suelen asociarse a la hambruna, las guerras o situaciones de inmunosupresión (Hommel 1999). En ocasiones, tras la infección los pacientes no manifiestan ninguna sintomatología, por lo que es habitual encontrar personas asintomáticas en las regiones endémicas de leishmaniosis. Es interesante destacar que estas personas son inmunes al desarrollo de la enfermedad (Probst, Stromberg et al. 2001).

La infección por *L. infantum* (= *L. chagasi* (Mauricio, Stothard et al. 2000)) en el perro también puede provocar una grave leishmaniosis visceral canina (LVC) y, en algunos casos, se producen también leishmaniosis cutáneas. Como ocurre en humanos,

y aunque los perros son muy sensibles al desarrollo de las leishmaniosis, la sintomatología varía entre individuos. Así, las leishmaniosis caninas se clasifican como: sintomáticas (presencia de varios síntomas), oligosintomáticas (presencia de algún síntoma) y asintomáticas, donde la infección con el parásito no provoca ningún síntoma clínico. Las formas asintomáticas son de especial relevancia puesto que pueden evolucionar a formas sintomáticas al existir un periodo de tiempo entre la infección y el desarrollo de LVC. Sin embargo, también pueden ser animales resistentes, capaces de producir de forma natural una serie de respuestas inmunitarias de tipo celular que impiden la proliferación del parásito (Otranto, Paradies et al. 2009). Además, las leishmaniosis caninas no sólo constituyen un problema de sanidad veterinaria. Los perros infectados, independientemente de la sintomatología que presenten, actúan como reservorio del parásito para la infección a los seres humanos (da Costa-Val, Cavalcanti et al. 2007).

## Respuesta inmunitaria innata frente al parásito.

Tras la entrada del promastigote en el hospedador vertebrado se produce una rápida interacción entre éste y los mecanismos inmunológicos innatos del hospedador. El resultado de la infección dependerá de la interacción del parásito con el sistema inmunitario del hospedador y del tipo de respuesta inmunitaria que se genere.

### *Leishmania* y el sistema del complemento.

Tras la infección, los promastigotes son capaces de activar la vía clásica, la vía alternativa y la vía de las lectinas del sistema de complemento. Aunque un porcentaje de parásitos son lisados, otros son capaces de evadir la lisis mediada por este sistema e incluso aprovecharse de éste para llegar a su célula hospedadora.

En relación a la vía alternativa, algunas de las moléculas presentes en la superficie de los promastigotes son capaces de degradar los factores del complemento depositados en la membrana. Un ejemplo es la glicoproteína de superficie GP63 (proteasa muy abundante en las formas amastigotes) que escinde el C3b en su forma inactiva C3bi. Además, el LPG impide la penetración del complejo lítico C5b-C9 en la membrana celular (Sacks and Sher 2002). Los receptores de complemento CR1 y CR3, localizados en la superficie de diferentes células fagocíticas, interaccionan con ambas formas del factor (C3b o C3bi) asociado a la membrana del parásito, facilitando así la fagocitosis. Además, tanto el LPG como la GP63 interaccionan directamente con el

receptor CR3 (Brittingham, Morrison et al. 1995; Peters and Sacks 2006). Por otra parte, la interacción de anticuerpos naturales (IgM) circulantes en el hospedador con los promastigotes activa la vía clásica del complemento que es capaz de destruir a la mayor parte de los promastigotes. De nuevo, aquellos promastigotes no lisados son capaces de aprovechar el sistema en su beneficio, ya que estos mismos anticuerpos permiten la inmunoadherencia del parásito a eritrocitos (o plaquetas en el caso de mamíferos no primates). Esta interacción constituye un mecanismo evasivo ya que, en un tiempo inferior a la destrucción del parásito por el complemento, *Leishmania* es transferida a las células fagocíticas profesionales (Dominguez and Torano 1999; Dominguez, Moreno et al. 2002; Dominguez, Moreno et al. 2003). Por otra parte, se ha demostrado que la vía de las lectinas es capaz de activarse tanto en ausencia de anticuerpos como de C1. En esta vía las "MBL" (lectinas de unión a manosa), un tipo de receptor de reconocimiento de patógenos, se une a los azúcares presentes en la superficie del parásito. La unión de MBL al parásito promueve su fagocitosis, ya que puede funcionar como una opsonina. De esta forma los parásitos escapan del sistema del complemento y son captados por las células diana de la infección (Ambrosio and De Messias-Reason 2005).

### *Leishmania* y el neutrófilo.

Los neutrófilos son las primeras células que acuden al lugar de infección tras la inoculación de los parásitos. Son reclutados fundamentalmente por las proteínas de la saliva del insecto vector (Peters, Egen et al. 2008). Tras la llegada al lugar de infección se producen dos efectos contrapuestos (Figura 2). Por un lado, se induce la liberación del contenido de las "NETs" ("neutrophil extracellular traps") lo que provoca la destrucción de los parásitos (Guimaraes-Costa, Nascimento et al. 2009). Por otra parte, los neutrófilos tienen la capacidad de fagocitar a los promastigotes. Esta interacción puede ocurrir (cuando los parásitos están opsonizados) por la interacción del CR3 del neutrófilo con el C3bi depositado en la superficie del parásito u ocurrir por interacción directa de los receptores de reconocimiento de patrones (PRR) del neutrófilo y moléculas de la superficie del parásito aún no identificadas (Laufs, Muller et al. 2002). La forma de entrada es importante, ya que sólo los parásitos internalizados de forma no opsonizada pueden sobrevivir en el interior del neutrófilo (Laufs, Muller et al. 2002). Además, los parásitos generan un efecto inmunosupresor, modulando al alza la producción de TGF-β y a la baja la producción de TNF-α del neutrófilo infectado

(Laskay, van Zandbergen et al. 2008). En este entorno, el neutrófilo pasa a comportarse como un reservorio del parásito hasta su paso al macrófago. Es destacable señalar que estos efectos ocurren únicamente cuando el inóculo infectivo presenta, además de los promastigotes metacíclicos, promastigotes con marcadores de apoptosis que juegan este importante papel en la infección (Laskay, van Zandbergen et al. 2008). El control de la apoptosis, en el caso de los neutrófilos infectados, es esencial en los estadios iniciales de la infección. Los neutrófilos infectados por *Leishmania* presentan una mayor viabilidad que los no infectados al retrasarse su muerte por apoptosis (Laskay, van Zandbergen et al. 2008), las proteínas de la saliva del vector (que acompañan al inóculo) estimulan la muerte apoptótica de las células infectadas (Prates, Araujo-Santos et al. 2011). En cualquier caso, cuando los neutrófilos infectados comienzan a presentar marcadores de apoptosis en superficie, son fagocitados por macrófagos, internalizando los parásitos de forma silenciosa, proceso conocido por su símil histórico como "caballo de Troya" (van Zandbergen, Klinger et al. 2004; Peters and Sacks 2006). De forma alternativa, los promastigotes libres no fagocitados por los neutrófilos o liberados por éstos tras su muerte por apoptosis pueden infectar a los macrófagos en el entorno anti-inflamatorio asociado a la presencia de los neutrófilos infectados (Ritter, Frischknecht et al. 2009).

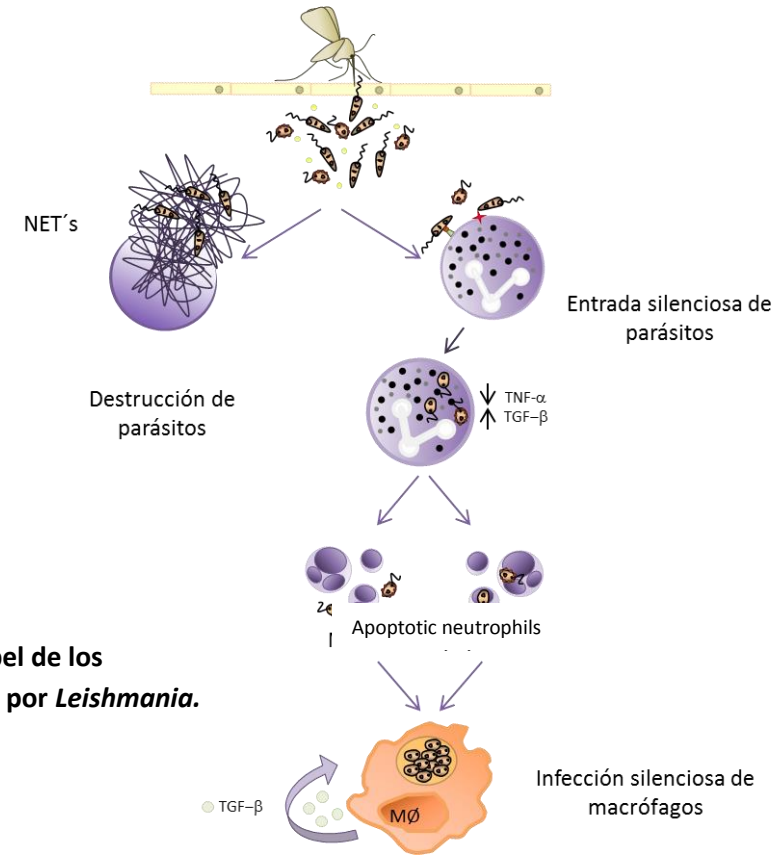


**Figura 2: Esquema del papel de los neutrófilos en la infección por *Leishmania.***

## *Leishmania* y el macrófago.

El macrófago es la principal célula hospedadora y cumple un doble papel. Por una parte, el establecimiento de la infección depende de que los promastigotes sean fagocitados por los macrófagos, y de que en el entorno ácido de la vacuola parasitófora (de origen lisosomal) se diferencien a amastigotes. Esta forma no flagelada es capaz de replicarse en el entorno ácido y rico en hidrolasas del interior del fagolisosoma (Cunningham 2002). Por otra parte, y como se describirá posteriormente, el macrófago es la principal célula implicada en la destrucción del parásito, siempre que se genere la respuesta inmunitaria adquirida adecuada.

Los promastigotes son capaces de infectar a los macrófagos empleando diferentes mecanismos. Así, los receptores de complemento CR1 y CR3 localizados en la superficie del macrófago, interaccionan con ambas formas del complemento C3b o C3bi asociado a la membrana del parásito facilitando su fagocitosis. Además del CR1 y CR3, la unión está también mediada por otros receptores del macrófago, como el receptor de fibronectina, los receptores manosa-fucosa y los receptores de la región constante de las inmunoglobulinas (Peters and Sacks 2006). Como se indicó

anteriormente, la fagocitosis de los neutrófilos apoptóticos infectados por *Leishmania* es una forma de infección.

El parásito, fundamentalmente por la acción de sus moléculas de superficie, es capaz de interferir en el correcto funcionamiento del macrófago para asegurar su propia supervivencia. Así, se ha descrito que en los macrófagos infectados se alteran las rutas de transducción de señales implicadas en diferentes procesos, lo que provoca un bloqueo de la activación tras la estimulación con citoquinas, una inhibición de los procesos de muerte celular por apoptosis, una incapacidad para secretar IL-12, una inhibición de la presentación antigénica, la síntesis de moléculas coestimuladoras e incluso una inhibición global de la síntesis de proteínas (revisado por (Sacks and Sher 2002; Peters and Sacks 2006; Shapira and Zinoviev 2011).

## Respuesta inmunitaria adquirida frente a *Leishmania*.

El conocimiento de las respuestas implicadas en la resistencia y/o susceptibilidad frente a la leishmaniosis, se ha desarrollado por el estudio de muestras de pacientes así como gracias al empleo de modelos experimentales de infección, siendo los modelos de ratón los más utilizados (Sacks and Noben-Trauth 2002; Scott, Artis et al. 2004). En relación a la infección del ratón por *L. major*, existen cepas susceptibles (BALB/c) y cepas resistentes (C57BL/6) (Sacks and Noben-Trauth 2002). La susceptibilidad que muestran los ratones BALB/c tras ser infectados por *L. major* se mantiene con otras especies cutaneotrópicas como *L. amazonensis* (Chang, Reed et al. 2003). Sin embargo, en el caso de la infección por *L. braziliensis*, los ratones BALB/c son capaces de controlar la infección si se produce de forma intradérmica (de Moura, Novais et al. 2005). En relación a las especies viscerotrópicas, los ratones BALB/c muestran una respuesta órgano específica. Tras la infección por *L. infantum* o *L. chagasi*, los ratones son capaces de eliminar los parásitos del hígado pero no son capaces de controlar la infección en el bazo (Melby, Yang et al. 2001; Ahmed, Colmenares et al. 2003).

Aunque existen diferencias en las repuestas generadas tras la infección con diferentes especies de *Leishmania*, y en diferentes cepas de ratón, se pueden sacar algunas conclusiones generales. Éstas se correlacionan, al menos en parte, con los aspectos inmunológicos asociados a la clínica tras la infección natural de los seres humanos y en el perro. En general, la inmunidad protectora se asocia al desarrollo de

respuestas inmunitarias inflamatorias controladas, mediadas principalmente por linfocitos $CD4^+$ Th1 y $CD8^+$ secretores de IFN-γ. Estas respuestas activan a los macrófagos. Así, la estimulación con IFN-γ resulta en una mejora del proceso de presentación antigénica, en la síntesis de TNF-α (citoquina que actúa de forma coordinada con el IFN-γ para el mantenimiento del estado activado del macrófago) y en la producción de óxido nítrico (NO), molécula con una gran capacidad leishmanicida (Bogdan, Gessner et al. 1996; Bogdan and Rollinghoff 1998). Este mecanismo de destrucción de los parásitos intracelulares es común para las leishmaniosis humanas, caninas y las desarrolladas experimentalmente en el ratón. Por otro lado, las respuestas Th2 y la producción de anticuerpos por los linfocitos B, así como las respuestas inmunosupresoras mediadas por IL-10, se relacionan con la susceptibilidad a la infección y el desarrollo de patología (Gradoni 2001; McMahon-Pratt and Alexander 2004).

Las células dendríticas son las encargadas de captar los parásitos y llevarlos hasta los ganglios linfáticos para presentar los antígenos del parásito directamente a las células T (Ritter, Frischknecht et al. 2009) (Figura 3). Dentro de la población de células dendríticas, las responsables de la migración hacia los ganglios linfáticos parecen ser las células dendríticas dérmicas o DDC. Este subtipo celular tiene más capacidad de migración que las células de Langerhans presentes en la epidermis (Ng, Hsu et al. 2008). También es importante mencionar que la captura de parásitos por parte de las células dendríticas no está influenciada por la presencia de neutrófilos infectados, al contrario de lo que ocurre con los macrófagos (Ng, Hsu et al. 2008). Las células dendríticas son las responsables, a través de la presentación de antígenos de *Leishmania*, de la diferenciación de los linfocitos Th precursores en células Th1 o en células Th2 (Sacks and Noben-Trauth 2002; Gumy, Louis et al. 2004). Esta diferenciación depende en gran medida de las señales coestimuladoras y el ambiente de citoquinas que rodean a las células dendríticas en el momento de la presentación antigénica. Así, en presencia de IL-4, IL-10 o TGF-β las células T se diferencian en células Th2, mientras que en presencia de IL-12 se diferencian en Th1 (Sacks and Noben-Trauth 2002; Sher, Pearce et al. 2003).

El tipo de respuesta resulta fundamental para la susceptibilidad o la resistencia a la infección. Por ejemplo, tras la infección de ratones BALB/c por *L. major* se produce una lesión cutánea progresiva y una diseminación de los parásitos a los órganos

internos, concomitante a la generación de una fuerte respuesta humoral mediada por linfocitos T $CD4^+$ del tipo Th2, con producción parásito específica de IL-4 e IL-13 (Reiner and Locksley 1995). La producción de ambas citoquinas está asociada a la progresión de la infección. Así, los ratones BALB/c "knock out" para la IL-4 son resistentes a la infección de algunas cepas de *L. major,* aunque el grado de resistencia aumenta si la deficiencia es del receptor de la IL-4 (compartido en su cadena alfa por ambas citoquinas) (Noben-Trauth, Paul et al. 1999). Por otra parte, la sobreexpresión de la IL-13 en ratones C57BL/6 los hace susceptibles a la infección por *L. major* (Matthews, Emson et al. 2000). Los linfocitos Th2 colaboran en la activación de los linfocitos B para la producción de una gran cantidad de anticuerpos del isotipo IgG1 dirigidos específicamente hacia muchas de las proteínas del parásito (Coffman 1993).

Por el contrario, la infección con *L. major* en ratones C57BL/6 induce la formación de lesiones inflamatorias similares a las observadas en humanos, que curan espontáneamente (Belkaid, Mendez et al. 2000). La lesión inflamatoria desaparece tras la activación de respuestas celulares del tipo $CD4^+$ Th1 y $CD8^+$ productoras de IFN-γ (Belkaid, Von Stebut et al. 2002). Estas respuestas celulares se correlacionan con la producción de leves respuestas humorales productoras de anticuerpos del isotipo IgG2a frente a diferentes antígenos parasitarios (Coffman 1993). La relación Th1-resistencia y Th2-susceptibilidad es extrapolable a las infecciones naturales. Así, los pacientes resistentes o recuperados de leishmaniosis muestran una reacción de hipersensibilidad de tipo retardada o DTH, positiva y poseen linfocitos T $CD4^+$ productores de IFN-γ frente a las proteínas del parásito (Kemp, Kemp et al. 1999; Probst, Stromberg et al. 2001; Khalil, Ayed et al. 2005). Por otra parte, los pacientes que presentan manifestaciones clínicas son DTH negativos (Crescente, Silveira et al. 2009) y presentan una serología positiva frente a las proteínas del parásito (Kar 1995). Finalmente, los anticuerpos generados frente a las proteínas del parásito forman complejos inmunes responsables de la patología en diferentes tejidos: sistema nervioso central (Garcia-Alonso, Nieto et al. 1996) y riñón (Mancianti, Poli et al. 1989; Nieto, Barrera et al. 1992; Nieto, Navarrete et al. 1992; Lopez, Lucena et al. 1996). Una excepción son los pacientes de LMC, ya que en sus lesiones se detecta una elevada producción de IFN-γ (Gomes-Silva, de Cassia Bittar et al. 2007).

La IL-10, producida por diferentes tipos celulares (linfocitos B, macrófagos infectados, así como diferentes tipos de linfocitos T (Moore, de Waal Malefyt et al.

2001)), juega un papel importante en la infección. Por un lado, está relacionada con la susceptibilidad. Así, los ratones BALB/c tratados con anticuerpos frente al receptor de la IL-10 son altamente resistentes a la infección con *L. major* (Kane and Mosser 2001; Noben-Trauth, Lira et al. 2003). En los modelos de infección con *L. infantum*, la cronificación esplénica depende de la síntesis de IL-10, ya que se ha comprobado que ratones BALB/c o C57BL/6 tratados con un anticuerpo bloqueante del receptor de IL-10, o cepas transgénicas deficientes en el gen de la IL-10, son capaces de destruir los parásitos presentes en el bazo (Murphy, Wille et al. 2001; Murray, Lu et al. 2002; Awasthi, Mathur et al. 2004; Garg and Dube 2006). Además, la interacción de los macrófagos con los complejos inmunes formados por la interacción entre los antígenos del parásito y los abundantes anticuerpos generados en el hospedador inducen la síntesis de IL-10 y reprimen la síntesis de IL-12 por parte de los macrófagos (Miles, Conrad et al. 2005). Todos estos datos relacionan la producción de IL-10 con la susceptibilidad en los modelos de ratón. Esta relación también existe en las leishmaniosis humanas y ha sido estudiada con detalle en pacientes con LV. Éstos presentan niveles elevados de IL-10 en el suero y una elevada expresión de los ARNm de IL-10 en los tejidos afectados (Caldas, Favali et al. 2005; Nylen and Sacks 2007). La IL-10 es producida por linfocitos T reguladores adaptativos (Tr1: $CD4^{+}Foxp3^{-}$) (Nylen, Maurya et al. 2007) e incluso por linfocitos Th1 específicos de los antígenos de *Leishmania* (Anderson, Oukka et al. 2007). Todos estos datos relacionan claramente a la IL-10 y a su papel anti-inflamatorio con la susceptibilidad a la infección por *Leishmania*. Sin embargo, existen evidencias que indican que esta citoquina puede jugar también un importante papel en la resistencia a la reinfección. En el modelo *L. major*-C57BL/6 se ha asociado la producción de IL-10 por células T reguladoras naturales ($CD4^{+}$ $Foxp3^{+}$) con el control de la respuesta efectora Th1 al final de la fase de resolución. Este control, que ocurre antes de la completa esterilización de los tejidos afectados conduce a la cronificación de la infección (Belkaid, Hoffmann et al. 2001; Belkaid, Piccirillo et al. 2002). La cronificación es importante, ya que la persistencia del parásito en el organismo se ha correlacionado con la protección a la reinfección, fundamentalmente por el mantenimiento de linfocitos T efectores frente a las proteínas del parásito (Uzonna, Wei et al. 2001; Scott, Artis et al. 2004; Okwor and Uzonna 2008). Finalmente, la alta relación IFN-γ/IL-10 encontrada en las lesiones se ha tomado como una evidencia de que la producción de IL-10 en pacientes con LMC puede tener un efecto favorable

controlando el proceso inflamatorio generado en la zona de la lesión (Gomes-Silva, de Cassia Bittar et al. 2007).

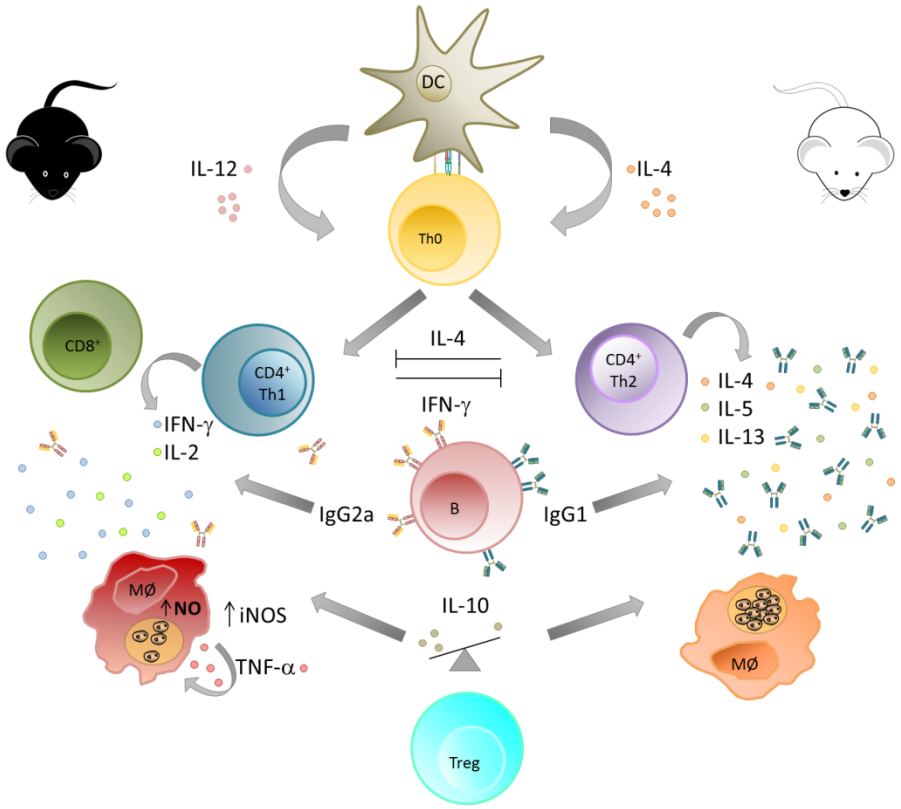


**Figura 3: Esquema de la respuesta inmune adquirida frente a *Leishmania*.**

## Tratamiento y diagnóstico.

Hoy en día, debido a la falta de vacunas frente a las leishmaniosis humanas, el único modo de combatir la enfermedad es mediante el empleo de fármacos. Los derivados pentavalentes de antimonio son, de forma general, el primer tipo de fármaco que se administra a los pacientes. Los más comunes son el antimoniato de meglumina (Glucantime®) y el estibogluconato sódico (Pentostam®). Estos compuestos se usan desde principios del siglo XX para tratar la leishmaniosis y están basados en la toxicidad del antimonio (revisado en (Gebel 1997; Frezard, Demicheli et al. 2009)). Su administración puede producir graves efectos secundarios. Otros fármacos leishmanicidas muy empleados son el isocianato de pentamidina y la anfotericina B (revisado en (Mondal, Bhattacharya et al. 2010)). Ambos se usan como alternativa cuando no se pueden administrar a los pacientes antimoniales pentavalentes, o en caso de que la terapia con éstos no sea efectiva. La miltefosina y la sitamaquina son otras alternativas al tratamiento. Tienen la ventaja de que pueden ser administrados por vía

oral mientras que los tres fármacos anteriores se administran por vía intravenosa o intramuscular.

El correcto diagnóstico de las leishmaniosis humanas y caninas es en la actualidad un problema no resuelto completamente. Es importante realizar un correcto diagnóstico tanto para confirmar la enfermedad en los pacientes sintomáticos como para iniciar los protocolos de tratamiento en los pacientes oligosintomáticos o asintomáticos que puedan evolucionar a formas clínicas. En el caso de la leishmaniosis cutánea, el diagnóstico clínico se complementa con un diagnóstico parasitológico. La detección de los parásitos se realiza fundamentalmente de forma directa mediante microscopía óptica en tinciones de raspados, biopsias o aspiraciones de las lesiones. Además, este material puede ser utilizado para el establecimiento de cultivos de parásitos y/o para detectar la presencia del parásito por técnicas de diagnóstico molecular (PCR) basadas en la amplificación de secuencias específicas del parásito (Chappuis, Sundar et al. 2007). En los casos de leishmaniosis visceral, la detección directa del parásito se realiza tras la toma de muestras por punción esplénica, en aspirados de médula ósea o en biopsias de ganglios linfáticos. Dado que estos procedimientos son muy invasivos la OMS recomienda el empleo de técnicas basadas en la detección de anticuerpos circulantes frente a proteínas del parásito. Estas técnicas se emplean para diagnosticar LV tanto humanas como caninas y están sustentadas en la evidencia experimental que demuestra que los pacientes con LV presentan un elevado título de anticuerpos contra antígenos del parásito. Estos anticuerpos pueden mantenerse temporalmente durante el tratamiento aunque tienden a disminuir tras la cura clínica. Las técnicas más utilizadas son la inmunofluorescencia indirecta (IFI), técnicas de "western blot", inmuno-cromatografía y ensayos de ELISA (Srividya, Kulshrestha et al. 2012). Como muestra antigénica se pueden emplear extractos totales del parásito, fracciones del parásito o antígenos individuales. La sensibilidad y especificidad de los sistemas de serodiagnóstico depende principalmente del antígeno empleado. En la mayoría de estas técnicas se suele utilizar en primer lugar un extracto de proteínas solubles del parásito (SLA), que permite obtener elevados valores de sensibilidad, sobre todo en las formas viscerales sintomáticas. Sin embargo, en las formas oligosintomáticas o asintomáticas de LV la sensibilidad de los diagnósticos basados en proteínas solubles puede ser muy variable, así como en las formas cutáneas y mucosas (Maalej, Chenik et al. 2003). El mayor inconveniente del SLA es su especificidad, ya que existen reacciones cruzadas entre las proteínas solubles de *Leishmania* y el suero de individuos infectados con otros

patógenos, especialmente con *Trypanosoma cruzi*, e incluso de pacientes con enfermedades autoinmunes (Ferreira Ede, de Lana et al. 2007; Porrozzi, Santos da Costa et al. 2007).

Como alternativa al empleo de SLA se han utilizado fracciones purificadas del parásito, como proteínas de membrana o proteínas intracelulares, y proteínas recombinantes (revisado en (Soto, Ramírez et al. 2009). En el caso de las leishmaniosis caninas, ninguna de las proteínas recombinantes ensayadas hasta la fecha ha permitido el desarrollo de un sistema de diagnóstico que presente el grado de sensibilidad conseguido por las proteínas totales (Maalej, Chenik et al. 2003; Porrozzi, Santos da Costa et al. 2007). Esto se debe al elevado grado de variabilidad en la respuesta humoral de los pacientes y los perros infectados frente a las proteínas individuales del parásito (Porrozzi, Santos da Costa et al. 2007; Goto, Howard et al. 2009). Los resultados pueden mejorar al emplear una mezcla de proteínas recombinantes, o proteínas recombinantes quiméricas en la que se fusionan los determinantes antigénicos de diferentes proteínas del parásito (Soto, Requena et al. 1998; Boarino, Scalone et al. 2005; Porrozzi, Santos da Costa et al. 2007). Como excepción, se ha desarrollado un test comercial es capaz de diagnosticar el 100% de los casos de LV sintomática humana basado en una proteína recombinante que contiene el extremo carboxilo terminal de la kinesina (rK39) (Badaro, Benson et al. 1996; Singh, Kumari et al. 2002). Este sistema, sólo puede emplearse para el diagnóstico de las formas sintomáticas humanas ya que su sensibilidad disminuye cuando se emplea en pacientes asintomáticos y para el diagnóstico del resto de leishmaniosis humanas y/o caninas (Badaro, Benson et al. 1996).

Existe una prueba inmunológica complementaria al serodiagnóstico, el test de Montenegro, que es muy utilizado para comprobar si un paciente (humano o canino) ha generado una respuesta celular frente a *Leishmania* (hipersensibilidad retardada). La prueba consiste en la inoculación intradérmica de una suspensión de promastigotes inactivados en solución fenólica. Si el resultado es positivo, tras 48 o 72 horas aparece una inflamación y endurecimiento de la zona donde se realizó la inyección. Generalmente, el test es positivo cuando se ha producido una cura bien sea de forma natural o tras el tratamiento, mientras que durante la fase activa de la enfermedad resulta negativo (Maia and Campino 2008).

## Vacunas frente a la leishmaniosis.

La vacunación parece ser la mejor opción en el proceso de control de la leishmaniosis ya que los individuos que se recuperan de la enfermedad son inmunes a posteriores infecciones. La inmunidad que sigue a la recuperación de la leishmaniosis deriva de la inducción de una respuesta mediada por linfocitos T, que lleva consigo la producción de citoquinas inflamatorias que activan a los macrófagos, provocando la destrucción de los parásitos. La inmunidad que se produce en un segundo contacto con el parásito debe estar mediada por células T memoria producidas durante el primer contacto con los antígenos del parásito. Estas células hacen que la respuesta frente a siguientes infecciones sea más rápida y específica. Dentro de este grupo de células se pueden diferenciar dos tipos, las células T memoria centrales (Tcm) y las células T memoria efectoras (Tem). Las Tem tienen la capacidad de responder rápidamente a un nuevo contacto con los antígenos del patógeno (Okwor and Uzonna 2008). La memoria inmunológica en los casos de infección se mantiene, probablemente, por la presencia persistente del parásito en el huésped por medio de un proceso llamado inmunidad concomitante (Aebischer, Moody et al. 1993).

### Primera generación de vacunas.

La leishmanización fue el primer método utilizado para producir una inmunidad duradera frente a *Leishmania*. Esta estrategia consiste en la inoculación de parásitos vivos (formas cutaneotrópicas) en zonas poco expuestas, como las nalgas o el interior de los brazos. De esta forma se pretende evitar la infección natural y la posible aparición de lesiones desfigurantes en zonas más visibles como la cara (Khamesipour, Abbasi et al. 2012). La leishmanización provoca una lesión cutánea capaz de auto-curarse. Esta estrategia no está recomendada por la OMS ya que se han reportado casos de individuos con lesiones recurrentes y de difícil curación. Como alternativa a la leishmanización se propuso comprobar si la vacunación con parásitos muertos era eficaz y segura. Se han llevado a cabo varios ensayos de vacunación. El primero de ellos se realizó en Brasil donde se desarrolló una vacuna basada en 5 especies diferentes de *Leishmania*. Esta vacuna, aunque inmunogénica y segura, sólo consiguió un 50% de protección. Otra aproximación fue la vacuna denominada Leishvacin®, compuesta únicamente por *L. amazonensis*, que fue probada en ensayos clínicos en Colombia y Ecuador entre otros países y, aunque resultó ser segura, no confería protección (Nagill and Kaur 2011). Estudios en modelos de ratón permitieron comprobar que la respuesta inmunitaria

generada por la leishmanización es cualitativamente diferente a aquella que se observa tras una vacunación con parásitos muertos. En el primer caso, se induce una rápida respuesta proinflamatoria mediada por IFN-γ, mientras que los parásitos muertos activan la producción de IL-4 (Okwor, Liu et al. 2009). Sin embargo, administrados junto con adyuvantes de tipo Th1, como los oligodeoxirribonucleótidos con motivos CpG no metilados (CpG-ODN), son capaces de conferir protección, si bien ésta no es de larga duración (Okwor, Liu et al. 2009).

Otra estrategia ha sido el uso de parásitos atenuados capaces de infectar, pero no de producir patología. Los primeros parásitos atenuados se produjeron mediante técnicas como la radiación gamma o el mantenimiento en cultivo durante largos periodos de tiempo, que generaban mutaciones indefinidas (Nagill and Kaur 2011). En la actualidad se investiga con el empleo de parásitos modificados genéticamente a los que se les ha añadido o eliminado algún gen que les impida tener gran persistencia en el huésped (Okwor and Uzonna 2009; Carrion, Folgueira et al. 2011). En algunos casos estos parásitos son capaces de recuperar su infectividad mediante mecanismos de compensación poco conocidos, volviéndolos peligrosos en su uso como vacunas (Okwor and Uzonna 2009). Alternativamente, se han desarrollado recientemente vacunas basadas en especies de *Leishmania* no patógenas genéticamente modificadas que sobre-expresan algún producto (Mizbani, Taheri et al. 2009; Saljoughian, Taheri et al. 2013) o incluso bacterias como *Salmonella* (Yang, Fairweather et al. 1990) o virus (*Vaccinia* y adenovirus) recombinantes que expresan proteínas del parásito (Palatnik-de-Sousa 2008).

### Vacunas de segunda generación: extractos parasitarios o proteínas recombinantes.

En la actualidad, muchas de las vacunas que se están ensayando están constituidas por extractos del parásito o por proteínas recombinantes. Algunas de las proteínas más estudiadas son aquellas que se encuentran en la superficie del parásito. Entre éstas podemos destacar la glicoproteína GP63 (Nagill and Kaur 2011) y un extracto de moléculas de superficie conocido como "FML" (Fucose-Manose Ligand) comercializado en Brasil con el nombre de Leishmune® (Borja-Cabrera, Cruz Mendes et al. 2004). Existe otra vacuna licenciada en Brasil frente a la leishmaniosis canina, basada en el antígeno A2 (una proteína específica de las formas amastigotes) denominada (Leish-Tec®) (Fernandes, Costa et al. 2008). Finalmente, a principios del

2012 se ha comercializado la primera vacuna contra la leishmaniosis canina en Europa, CaniLeish, basada en antígenos secretados y excretados del parásito (Moreno, Vouldoukis et al. 2012; Moreno, Vouldoukis et al. 2014).

Muchas proteínas intracelulares pertenecientes a familias de proteínas conservadas resultan antigénicas durante la infección del parásito (proteínas de choque térmico, histonas, proteínas del ribosoma) (Requena, Alonso et al. 2000). Casi todas ellas generan respuestas humorales no protectoras mediadas por linfocitos Th2, por lo que han sido consideradas como determinantes patoantigénicos, ya que actúan a modo de toxinas que promueven fuertes respuestas humorales ineficaces y patológicas (Chang and McGwire 2002; Chang, Reed et al. 2003). Pese a que estas proteínas actúen como patoantígenos durante las leishmaniosis, no puede descartarse su empleo como vacunas. De hecho, en pacientes resistentes o recuperados de la infección, se generan respuestas celulares protectoras específicas frente a estos antígenos (Probst, Skeiky et al. 1997). Por ello, la redirección de estas respuestas hacia la generación de respuestas Th1 empleando protocolos de inmunización adecuados es una estrategia para el desarrollo de vacunas contra la leishmaniosis (revisado en (Campos-Neto 2005; Soto, Ramírez et al. 2009)). Así, la elección del adyuvante es fundamental en estas estrategias vacunales. Los adyuvantes son moléculas que administradas junto con el antígeno hacen más efectiva o redirigen la respuesta inmunológica. Hoy en día se emplean diferentes tipos de adyuvantes capaces de producir las respuestas Th1 necesarias para prevenir la infección por *Leishmania.* A continuación se explica el mecanismo de acción de algunos de los adyuvantes más utilizados en las vacunas frente a *Leishmania*.

**CpG-ODN**: Son moléculas de ADN monocatenario o bicatenario con motivos CpG no metilados. La capacidad adyuvante de los motivos CpG depende de la estimulación directa de diversos tipos celulares del sistema inmunitario al interaccionar con el TLR9 ("Toll-like Receptor 9"). Los motivos CpG estimulan respuestas Th1 al generar la secreción de diversas citoquinas proinflamatorias: IL-12, TNF-α, IFN-γ, IFN-α (Rothenfusser, Tuma et al. 2002).

**Saponinas**: Son moléculas de carácter anfipático constituidas por una parte lipídica (terpenos o esteroides), y una parte hidrofílica (restos de azúcares). Estas moléculas estimulan respuestas Th1 y producen respuestas mediadas por linfocitos T citotóxicos (Sun, Xie et al. 2009).

**BCG**: Es un extracto atenuado de *Mycobacterium bovis* (la vacuna contra la tuberculosis). Se emplea como adjuvante en el tratamiento de algunos tipos de cáncer ya

que estimula la producción de células inflamatorias y citoquinas contra las células cancerígenas (Kresowik and Griffith 2009). La administración de BCG produce la proliferación de células T $CD4^+$ y un incremento de respuestas mediadas por IFN-γ (Ravn, Boesen et al. 1997).

**Monofosforil lípido A (MPL)**: deriva del lipopolisacárido (LPS) constituyente de la pared celular de bacterias Gram negativas y posee propiedades inmunoestimulantes. Se ha evaluado como adyuvante para promover la respuesta inmunológica frente a antígenos poco inmunogénicos por su interacción con el TLR4 (Tagliabue and Rappuoli 2008).

**Vacunas de ADN:** En 1990 se descubrió que la inyección de ADN plasmídico en solución acuosa era capaz de transformar células musculares (Wolff, Malone et al. 1990). A partir de entonces, esta técnica comenzó a utilizarse para la expresión *in vivo* de proteínas foráneas. El uso de los vectores recombinantes para la producción de vacunas ha sido muy estudiado en muchos tipos de enfermedades como aquellas causadas por virus, parásitos e incluso como vacuna contra algunos tipos de cáncer, ya que el ADN es capaz de producir anticuerpos y respuestas celulares mediadas tanto por linfocitos T $CD4^+$ como $CD8^+$ en modelos animales, frente a las proteínas que se encuentran codificadas por el vector (Donnelly, Ulmer et al. 1997). Por este motivo se han investigado este tipo de vacunas en el campo de las enfermedades infecciosas incluyendo *Leishmania* (Beaumier, Gillespie et al. 2013; Mutiso, Macharia et al. 2013). Estas vacunas y el análisis de su antigenicidad fueron la base para el diseño de los adyuvantes tipo CpG-ODN (Gurunathan, Klinman et al. 2000).

La capacidad para modular la respuesta inmunológica frente a las proteínas intracelulares conservadas empleando los citados adyuvantes ha permitido aprovechar las ventajas de este tipo de proteínas. Están presentes en las dos formas del parásito (promastigote y amastigote) y al ser proteínas con una función celular indispensable, no pueden ser modificadas ni sustituidas por otras moléculas para evadir la respuesta inmunológica generada contra ellas. Además, están muy conservadas entre las diferentes especies del parásito, por lo que podrían emplearse para generar vacunas frente a las diversas formas de leishmaniosis. A pesar de pertenecer a familias conservadas, la respuesta humoral y celular generada es específica de las proteínas de *Leishmania*, ya que los epítopos T y B se encuentran localizados en las regiones de secuencia divergentes entre las proteínas del parásito y las del hospedador (Requena,

Alonso et al. 2000; Probst, Stromberg et al. 2001; Iborra, Soto et al. 2004; Carmelo, Zurita et al. 2006; Iborra, Parody et al. 2008; de Oliveira, Nascimento et al. 2009). Esta especificidad indica que la respuesta se genera frente a las proteínas del parásito y no por procesos de autoinmunidad. Estas proteínas quedarían expuestas al sistema inmunológico del hospedador después de la infección, bien por la lisis de los promastigotes mediada por productos extracelulares de los neutrófilos, (Guimaraes-Costa, Nascimento et al. 2009) o bien por lisis mediada por el complemento (Dominguez, Moreno et al. 2003), permitiendo así un primer contacto de estos antígenos con las células presentadoras de antígeno en el sitio de infección. Posteriormente, la lisis de los amastigotes extracelulares o la lisis de los macrófagos infectados conteniendo antígenos de *Leishmania* permitiría mantener la estimulación de las respuestas dirigidas hacia ellas (Chang and McGwire 2002; Chang, Reed et al. 2003; de Oliveira, Nascimento et al. 2009).

## Los ribosomas como vacunas contra *Leishmania*.

Al comienzo de esta Tesis Doctoral existían algunas evidencias en el laboratorio de que el ribosoma es una partícula inmunogénica durante la infección por *Leishmania*. En algunos casos las respuestas se relacionaban con la resistencia a la infección y en otros casos con la susceptibilidad. Así, la proteína S4 de *Leishmania* es un antígeno reconocido por células T $CD4^+$ productoras de IFN-γ en pacientes DTH positivos residentes en zonas endémicas para leishmaniosis visceral y que nunca desarrollaron patología, así como en pacientes recuperados de leishmaniosis cutánea (Probst, Stromberg et al. 2001). Por el contrario, la proteína ribosómica P0 genera fuertes respuestas humorales en pacientes (Requena, Alonso et al. 2000) y la proteína S3 provoca la expansión policlonal de células B inhibiendo las células T tras la infección (Cordeiro-Da-Silva, Borges et al. 2001).

En relación al desarrollo de vacunas basadas en proteínas ribosómicas, la modulación de la respuesta inmunológica frente a alguna de ellas es capaz de generar protección frente a la infección del parásito. En el caso de la proteína ácida P0, la vacunación genética (plásmidos de expresión eucariota y/o administración de la proteína recombinante junto a CpG-ODN) consiguió generar respuestas celulares capaces de inducir un alto grado de protección frente a *L. major* en ratones resistentes C57BL/6 infectados con *L. major* (Iborra, Carrion et al. 2005) y retrasar la progresión de la patología en ratones BALB/c (Iborra, Soto et al. 2003; Iborra, Carrion et al. 2005). Esta

protección, se correlacionó con la generación de respuestas Th1 específicas frente a la proteína P0 (en ambos modelos) y con el retraso en la generación de respuestas humorales frente a los antígenos parasitarios en el modelo susceptible. Empleando el mismo modelo susceptible de LC, las proteínas ribosómicas L22 y S19 fueron identificadas como moléculas protectoras en un análisis masivo de vacunas de ADN (Stober, Lange et al. 2006). De forma similar, y mediante la inmunización de subgenotecas de *Leishmania* para la búsqueda de proteínas protectoras, se observó que la vacunación genética conjunta (vacunas de ADN) de las proteínas ribosómicas S14, S13, S16, S18, L8 y L28 indujo una inmunidad parcial frente a la LV en ratones BALB/c (Melby, Ogden et al. 2000).

Con estos antecedentes, el grupo donde se realizó esta Tesis Doctoral demostró que una preparación de proteínas del ribosoma era capaz de inducir respuestas protectoras frente a la infección por *L. major* tanto en ratones BALB/c como en ratones C57BL/6 cuando se administraba en presencia de CpG-ODN (Iborra, Parody et al. 2008). La inmunidad se asoció a la generación de respuestas mediadas por IFN-γ (en los dos modelos) junto al control de las respuestas asociadas con la patología (producción de IL-4 e IL-10 frente a los antígenos parasitarios). Esta publicación constituyó el punto de partida de esta Tesis Doctoral.

# MATERIALES y RESULTADOS

MATERIAIS, MÉTODOS E RESULTADOS

MATÉRIAUX, MÉTHODES ET RÉSULTATS

MATERIALIEN, METHODEN UND ERGEBNISSE

МАТЕРИАЛЫ, МЕТОДЫ И РЕЗУЛЬТАТЫ

MATERIALE, METODE ŞI REZULTATE

MATERIALI, METODI I REZULTATI

MATERIALER, METODER OG RESULTATER

MATERIAALIT, MENETELMÄT JA TULOKSET

MATERIALER, METODER OCH RESULTAT

MATERIALE, METODE ȘI REZULTATE

MATERIAALID, MEETODID JA TULEMUSED

MATERIJALI, METODE I REZULTATI

MATERIJALI, METODE I REZULTATI

MATERIALY, METODY A VÝSLEDKY

MATERIÁLY, METODY A VÝSLEDKY

MATERIAŁY, METODY I WYNIKI

ANYAGOK, MÓDSZEREK ÉS EREDMÉNYEK

MATERIALER, METODER OG RESULTATER

MATERIALEN, METHODEN EN RESULTATEN

MATERIALEN, METHODEN EN RESULTATEN

ΥΛΙΚΑ, ΜΕΘΟΔΟΙ ΚΑΙ ΑΠΟΤΕΛΕΣΜΑΤΑ

MATERIALAK, METODOAK ETA EMAITZAK

MATERIALS, MÈTODES I RESULTATS

วัสดุวิธีการและผล

材料、方法および結果

材料，方法和结果

مواد، روش‌ها و نتایج

مواد، روش‌ها و نتایج

مواد، روش‌ها و نتایج

مواد، روش‌ها و نتایج

Materials, Methods and Results

Материали, методи и резултати

Материалы, Методы и результаты

উপকরণ, পদ্ধতি ও ফলাফল

Matériaux, méthodes et résultats

**Resumen del artículo: "Specific serodiagnosis of canine visceral leishmaniasis using *Leishmania species* ribosomal protein extracts". Clinical and Vaccine Immunology (CVI), 2009. 16(12):1774-1780.**

El correcto diagnóstico de la leishmaniosis canina (LVC) sigue siendo un campo de investigación en desarrollo. Existen dos formas de diagnóstico de la LVC: la detección del parásito o la determinación de la presencia de respuestas de anticuerpos contra las proteínas inmunodominantes del mismo. A la hora de detectar la presencia de anticuerpos frente al parásito, los ensayos suelen basarse en extractos de proteínas totales (SLA). Esta fuente de antígeno, pese a tener una elevada sensibilidad, presenta la limitación de su especificidad, ya que puede darse una reactividad cruzada entre el SLA y los anticuerpos que se encuentran en el suero de animales infectados por otros patógenos o afectados por enfermedades autoinmunes. Un problema adicional del SLA es que, en muchos casos, la seropositividad aparece asociada con la aparición de síntomas clínicos, por lo que la serología sólo confirma la enfermedad y no puede emplearse en el tratamiento temprano. Finalmente, el empleo de vacunas anti-*Leishmania* de uso veterinario ha abierto la necesidad de distinguir los anticuerpos generados por la vacunación de los producidos por una infección.

En este trabajo se analizó el potencial diagnóstico de una fracción de ribosomas de *Leishmania* (LRP) purificados a partir de las formas promastigotes empleando una colección variada de sueros de perro:

- Sueros de animales con LVC sintomática, oligosintomática y asintomática.
- Sueros de animales sanos.
- Sueros de animales infectados con otros patógenos.
- Suero de animales vacunados con Leishmnune® y Leishtech®.

Para ello se realizaron análisis de ELISA, efectuándose un estudio comparativo entre LRP y SLA. Los resultados obtenidos demostraron que muchas de las proteínas del ribosoma de *Leishmania* son antigénicas (Figura 1; CVI) y que los ribosomas son reconocidos por el suero de animales infectados de forma independiente a sus manifestaciones clínicas (Figura 2; CVI). También cabe destacar que el extracto de ribosomas de *Leishmania* presenta mayor especificidad de reconocimiento que el SLA

en animales infectados con otros patógenos, así como un similar patrón de reconocimiento cuando se analizan sueros de perros vacunados con vacunas comerciales empleadas en Brasil (Figura 3; CVI). Estos datos sugieren que el ribosoma puede ser una herramienta atractiva para el desarrollo de sistemas de diagnóstico serológico de la LVC. La aparición de anticuerpos contra las proteínas del ribosoma no parece ser una consecuencia de la desregulación del sistema inmunitario al no estar asociada a las formas más extremas de la patología. Estos resultados avalan la hipótesis de que los ribosomas son partículas inmunogénicas reconocidas de forma temprana tras la infección por el sistema inmunológico del hospedador vertebrado.

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# Specific Serodiagnosis of Canine Visceral Leishmaniasis Using *Leishmania* Species Ribosomal Protein Extracts▽

Eduardo A. F. Coelho,[1,2] Laura Ramírez,[3] Mariana A. F. Costa,[1] Vinicio T. S. Coelho,[1] Vivian T. Martins,[1] Miguel A. Chávez-Fumagalli,[5] Dulcilene M. Oliveira,[1] Carlos A. P. Tavares,[1] Pedro Bonay,[3] Carlos Gómez Nieto,[4] Daniel R. Abánades,[3] Carlos Alonso,[3] and Manuel Soto[3]*

*Departamento de Bioquímica e Imunologia, ICB,[1] and Departmento de Patologia Clínica, Coltec,[2] Universidade Federal de Minas Gerais, 31.270-901, Belo Horizonte, Minas Gerais, Brazil; Centro de Biología Molecular Severo Ochoa, CSIC-UAM, Departamento de Biología Molecular, Universidad Autónoma de Madrid, Madrid 28049, Spain[3]; Unidad de Parasitología y Enfermedades Parasitarias, Universidad de Extremadura, Cáceres, Spain[4]; and Departamento de Farmacologia Bioquímica e Molecular, ICB, Universidade Federal de Minas Gerais, 31.270-901, Belo Horizonte, Minas Gerais, Brazil[5]*

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**In the present work, we have analyzed the antigenicity of *Leishmania* species ribosomal proteins (LRPs). To accomplish this, *Leishmania infantum* ribosomes were biochemically purified from promastigote cytosolic extracts, and their reactivities were analyzed by using the sera from dogs naturally infected with *L. infantum*. Since antibodies reacting against different ribosomal proteins were observed in all the serum samples obtained from dogs with symptomatic visceral leishmaniasis tested, we have analyzed the potential usefulness of the LRP extracts in the development of an enzyme-linked immunosorbent assay (ELISA) for the serodiagnosis of canine visceral leishmaniasis (CVL) in an area of Brazil where visceral leishmaniasis is endemic due to infection by *Leishmania chagasi*. A comparative ELISA with crude soluble *Leishmania chagasi* antigen (SLA) and *L. infantum* LRPs was performed. LRP- and SLA-based ELISAs gave similar sensitivities for the diagnosis of symptomatic CVL, but the LRP extract provided a very high sensitivity for the detection of oligosymptomatic and asymptomatic dogs. In addition, an LRP-based ELISA showed a higher specificity when the sera from dogs harboring other infections were included in the analysis. The LRP antigen displayed no cross-reactivity with sera from dogs that had any of the other diseases tested, notably, Chagas' disease. Our findings suggest that LRPs are a potential tool for the diagnosis of CVL and will be particularly useful for the diagnosis of asymptomatic CVL.**

Canine visceral leishmaniasis (CVL) is an important emerging zoonosis in countries around the Mediterranean Basin, the Middle East, and Latin America (26). This severe disease is caused by *Leishmania infantum* in the Mediterranean area and in the Middle East and Asian countries and is caused by *Leishmania chagasi* in Latin America (26, 27). Due to their genotypic relationships, both species causing CVL in different continents can be considered to be identical (32).

Upon infection, dogs can develop different forms of the disease: asymptomatic, oligosymptomatic, or symptomatic (5). Symptomatic infection results in death; and its clinical manifestations include cutaneous alterations, such as alopecia, dermatitis, and onychogryphosis (4, 16), and also visceral manifestations with splenic, renal, hepatic, and cerebral alterations (22, 37). However, some of the infected dogs remain asymptomatic or develop a few mild symptoms and are classified as oligosymptomatic (5). CVL cannot be considered only a veterinary disease, since infected dogs (even asymptomatic ones) are the main domestic reservoir of the parasite for human infection (2). Thus, to reduce the frequency of transmission of *Leishmania* from dogs to humans, it is necessary to diagnose canine leishmaniasis as early as possible (35).

The presence of anti-*Leishmania*-specific antibodies in asymptomatic, oligosymptomatic, and symptomatic infected dogs (5, 12, 45) has allowed the development of serologic tests, including immunofluorescent antibody tests, Western blotting, immunochromatographic tests, and enzyme-linked immunosorbent assays (ELISAs) (for a review, see reference 29). The diagnosis of CVL by ELISAs based on crude soluble *Leishmania* antigens (SLAs) have shown a high degree of sensitivity but a low degree of specificity because of the antigenic relatedness of *Leishmania* and other pathogenic protozoa (21). As a strategy for the development of specific serodiagnostic tests for CVL, different parasite antigens were obtained as recombinant proteins (23, 30, 48). However, due to the high degree of variability in the humoral responses to different parasite antigens observed in infected dogs (24, 42), the efficient diagnosis of CVL based on recombinant proteins may require a mixture of recombinant proteins or the use of chimerical proteins containing several nonrelated parasite antigens (7, 42, 49). The specific diagnosis of CVL can also be developed by using preparations purified from the parasite (6, 9) or crude parasite fractions analyzed by Western blotting (1, 20).

Some of the parasite ribosomal constituents, such as the parasite acidic P proteins, induce strong humoral responses in dogs clinically infected with *L. infantum* (46). In addition,

* Corresponding author. Mailing address: Centro de Biología Molecular Severo Ochoa (CSIC-UAM), Departamento de Biología Molecular, Nicolás Cabrera 1, Campus de Cantoblanco, Universidad Autónoma de Madrid, Madrid 28049, Spain. Phone: 34 91 1964508. Fax: 34 91 1964420. E-mail: msoto@cbm.uam.es.
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*Leishmania* ribosomal proteins (LRPs) seem to be immunologically relevant molecules during murine experimental cutaneous leishmaniasis because high titers of antibodies recognizing the parasite ribosomal proteins were detected in sera from BALB/c mice infected with *L. major* (28). Here, we show that sera from dogs naturally infected with *L. infantum* showed reactivity against different ribosomal proteins by Western blotting. In addition, a serological evaluation of LRPs by ELISA with these sera revealed that LRP-based assays have a sensitivity similar to that of SLA-based assays for the diagnosis of symptomatic CVL. Thus, the ELISA technique was used to evaluate the diagnostic potential of LRP extracts in comparison with that of SLAs by using sera from asymptomatic, oligosymptomatic, and symptomatic *L. chagasi*-infected dogs as well as dogs infected with other protozoan parasites. We conclude that the ELISAs with LRPs have a better sensitivity and a higher specificity than the SLA-based assays for the diagnosis of CVL.

## MATERIALS AND METHODS

**Parasites.** *Leishmania chagasi* (MOM/BR/1970/BH46) and *L. infantum* (MCAN/ES/1996/BCN/150/MON-1) were grown at 24°C in Schneider's medium (Sigma, St. Louis, MO) supplemented with 20% heat-inactivated fetal bovine serum (Sigma), 20 mM L-glutamine, 200 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin at pH 7.4.

**Antigen preparation.** SLA was prepared from stationary-phase promastigotes of *L. chagasi* and *L. infantum*, as described previously (17). Briefly, $2 \times 10^8$ promastigotes per ml, in a volume of 5 ml, were washed three times in cold sterile phosphate-buffered saline (PBS). After six cycles of freezing and thawing followed by ultrasonication (GEX600 ultrasonic processor) by the use of five cycles of 30 s at 38 MHz, the suspension was centrifuged at 8,000 × *g* for 30 min at 4°C and the supernatant containing the SLA was collected. The protein concentration was estimated by the method of Bradford (8), and aliquots of 500 μl were stored at −70°C.

LRP was prepared from logarithmic-phase promastigotes of *L. infantum*, as described previously (28). Briefly, $1 \times 10^9$ promastigotes were harvested, washed twice in prechilled PBS, resuspended in 1 ml Nonidet P-40 lysis buffer (10 mM Tris HCl, pH 8.0, 150 mM NaCl, 1.5 mM $MgCl_2$, 0.5% Nonidet P-40), and pipetted up and down 10 times. After lysis, the samples were centrifuged on a Microfuge at 3,000 × *g* for 2 min at 4°C to pellet the nuclei. The supernatant was twice centrifuged on a Microfuge at 13,000 × *g* for 15 min at 4°C. The purified cytosolic supernatant was submitted to high-speed centrifugation at 90,000 rpm for 30 min at 4°C in a Beckman TL100.3 rotor. The crude ribosomal pellet was resuspended in buffer A (20 mM Tris-HCl, pH 7.4, 500 mM ammonium acetate, 100 mM $MgCl_2$, 5 mM β-mercaptoethanol) and centrifuged through a discontinuous sucrose gradient (20/40%) in buffer A at 90,000 rpm and 4°C in a TL100.3 rotor. The pellet of washed ribosomes was dissolved in PBS, sonicated, and stored at −70°C. The LRP concentration was estimated by the method of Bradford (8).

**Serum samples.** Serum samples were collected in Spain and Brazil. Serum samples were collected from 28 clinically symptomatic dogs with CVL in the Extremadura region of Spain. *L. infantum*-infected animals were clinically, immunologically, and parasitologically evaluated at the Department of Parasitology of the Veterinary School, Extremadura University, Cáceres, Spain. The animals were considered symptomatic when three or more of the following symptoms were present: loss of weight; alopecia; adenopathy; onychogryposis; hepatomegaly; conjunctivitis; and exfoliative dermatitis on the nose, tail, and ear tips. All serum samples were positive when they were tested by indirect immunofluorescence, as described previously (10). The presence of amastigote forms was confirmed by direct observation in popliteal and prescapular lymph nodes. Control sera were obtained from eight healthy animals that had been bred and maintained in an experimental kennel under defined conditions at the Department of Parasitology (Extremadura University).

Serum samples from 83 *L. chagasi*-infected dogs (44 clinically symptomatic, 17 oligosymptomatic, and 22 asymptomatic dogs) from the Belo Horizonte area, Minas Gerais, Brazil, were used. The animals were considered symptomatic when they presented with three or more of the clinical symptoms described above, were considered oligosymptomatic when only one or two symptoms were present, and were considered asymptomatic when the dogs were free from clinical symptoms. Diagnosis of the disease was defined when amastigotes were seen in Giemsa-stained smears of bone marrow aspirates or promastigotes were identified on culture of peripheral blood or bone marrow aspirates. Fourteen serum samples from dogs with other parasite infections were used to analyze cross-reactivity, as follows: *Toxoplasma gondii* ($n = 5$) and *Trypanosoma cruzi* ($n = 9$). These sera were provided by Evaldo Nascimento and Maria Norma Melo (Department of Parasitology, Institute of Biological Sciences, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil). Serum samples from 47 dogs that were living in regions where visceral leishmaniasis (VL) is endemic but that had no clinical signs or suspicion of CVL and that were negative after parasitological and serological tests constituted the control group. Serum samples from healthy dogs and from dogs vaccinated with the Leishmune vaccine ($n = 18$) or the Leishtec vaccine ($n = 23$) were used to analyze their reactivity with LRPs and the SLA.

**ELISA.** Microtiter immunoassay plates (Falcon) were coated with *L. infantum* or *L. chagasi* SLA or with *L. infantum* LRPs (each one at 0.5 μg/well) in coating buffer (pH 9.6) for 18 h at 4°C. A titration curve was prepared to determine the best protein concentration (for LRPs, 4 μg to 0.063 μg; for SLA, 2 μg to 0.031 μg) and the best antibody dilution to be used. Free binding sites were blocked with a PBS–0.05% Tween 20 (PBST) and 3% casein solution for 2 h at 37°C. After three washes with PBST, the plates were incubated with 100 μl of canine serum for 1 h at 37°C. Serum samples were diluted 1:200 in PBST and 0.3% casein. The plates were then washed seven times and incubated with horseradish peroxidase-conjugated 1:10,000 anti-dog immunoglobulin G (IgG) antibody (Sigma). The reaction developed through incubation with $H_2O_2$, *ortho*-phenylenediamine, and citrate-phosphate buffer (pH 5.0) for 30 min in the dark and was stopped by addition of 2 N $H_2O_2$. The optical densities were read at 492 nm in an ELISA microplate spectrophotometer (Spectra Max Plus; Molecular Devices, Concord, Ontario, Canada).

**Western blotting.** For Western blot analysis, *L. infantum* SLA (15 μg) and LRPs (15 μg) were resuspended in Laemmli's buffer and resolved in a 10% (SLA) or a 10 to 14% gradient (LRP) sodium dodecyl sulfate (SDS)-polyacrylamide gel with preparative combs by using a Bio-Rad (Hercules, CA) protein electrophoresis minigel system. In both cases, the gels were transferred to nitrocellulose membranes (GE Healthcare). The blots were probed individually with the sera (1:200) from dogs infected with *L. infantum* (by SDS-polyacrylamide gel electrophoresis). As the secondary antibody, horseradish peroxidase-conjugated anti-dog IgG (1:2,000) purchased from Nordic Immunological Laboratories (Tilburg, The Netherlands) was used.

**Statistical analysis.** Statistical analysis was performed with GraphPad Prism software (version 4 for Windows). The reactivities of the serum samples collected in Spain against SLA and LRPs were tested for significance by using the Mann-Whitney test. The reactivities of the serum samples collected in Brazil against SLA and LRPs were evaluated by means of the one-way analysis of variance nonparametric test (Kuskal-Wallis by using the Dunn posttest for multiple comparisons of groups). *P* values of $<0.05$ were considered statistically significant.

## RESULTS

**Antigenicity of LRPs during canine infection.** In order to analyze the antigenicity of the LRPs during canine infection, sera from 10 dogs clinically infected with *L. infantum* that recognized a large number of protein bands in the SLA extracts (Fig. 1A, SLA panel, lanes 4 to 13) were incubated with a nitrocellulose membrane containing the LRP extracts from this species. All serum samples from dogs with symptomatic CVL recognized the parasite ribosomal purified protein fraction (Fig. 1A, panel LRP, lanes 4 to 13). The sera from healthy dogs were negative, and only a few protein bands were slightly stained in the LRP and SLA preparations (Fig. 1A, lanes 1 to 3). Most of the serum samples from dogs with symptomatic CVL recognized several protein bands in both protein extracts, although the complexity and intensity of the recognition pattern were different between the serum samples from individual dogs. Despite the variability obtained, two immunodominant regions were observed in the LRP Western blot: 45- to 36-kDa and 25- to 22-kDa proteins. The immunoreactivities of sera

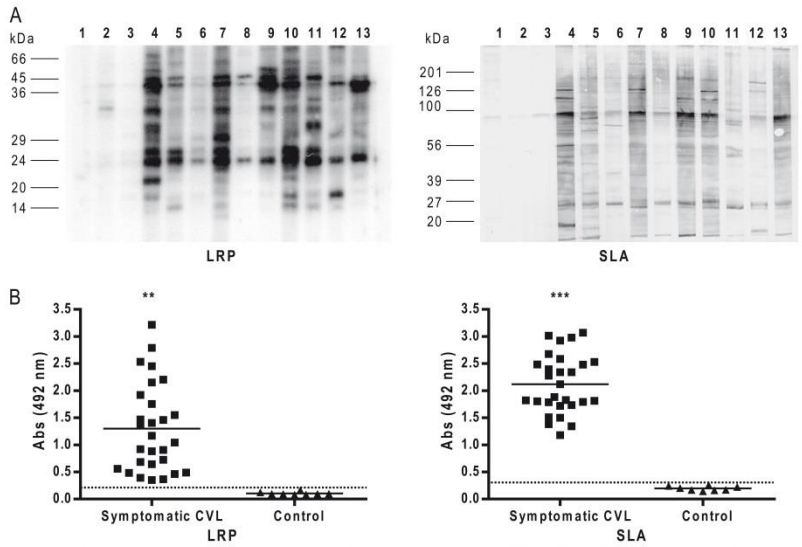


FIG. 1. (A) *Leishmania infantum* LRPs were electrophoresed on a linear 10 to 14% gradient SDS-polyacrylamide gel. *Leishmania infantum* SLA was electrophoresed on a 10% SDS-polyacrylamide gel. Both gels were transferred onto nitrocellulose membranes and incubated with sera from healthy dogs maintained in an experimental kennel under defined conditions (lanes 1 to 3) and sera from dogs naturally infected with *L. infantum* and with symptomatic CVL (lanes 4 to 13). The individual serum samples were used at a 1:200 dilution. A horseradish peroxidase-conjugated anti-dog IgG antibody was used as the secondary reagent. (B) ELISA reactivity of sera collected in Spain from dogs clinically infected with *L. infantum* and control sera with LRPs and SLA. Solid lines, mean values; dotted lines, cutoff value, defined as the mean optical density plus 3 standard deviations of the values obtained with sera from healthy controls. Abs, absorbance; **, $P < 0.01$; ***, $P < 0.001$.

from dogs clinically infected with *L. infantum* against LRP and SLA were measured by ELISA. Figure 1B shows the absorbance values for the sera from dogs with symptomatic CVL ($n = 28$) and the controls ($n = 8$) collected in Spain. For both protein preparations, sera from 100% of the dogs with symptomatic CVL showed absorbance values above the cutoff, and the differences in the mean value of the reactivity between the sera from dogs with symptomatic CVL and control sera were statistically significant.

**Comparison of LRPs and SLA for serodiagnosis of CVL.** Since antibodies reacting against LRP were observed in the sera from dogs with symptomatic CVL due to *L. infantum* infection, we analyzed whether the *L. infantum* LRP extract could be considered a valuable tool for the serodiagnosis of CVL in other regions of the world. For that purpose, we analyzed the reactivity against *L. infantum* LRPs and *L. chagasi* SLA of 130 canine serum samples collected in a region of Brazil that is endemic for CVL due to infection by *Leishmania chagasi*. Those samples included sera from dogs infected with *L. chagasi* with symptomatic infections ($n = 44$), oligosymptomatic infections ($n = 17$), and asymptomatic infections ($n = 22$), as well as 47 healthy dogs living in that area of endemicity. For both protein preparations, the differences between the mean of the absorbance values observed for sera from dogs with symptomatic CVL and healthy control sera were statistically significant ($P < 0.001$) (Fig. 2). The spectrum of the absorbance values from the LRPs and SLA were different, as the reactivity of sera from dogs with CVL against SLA was higher (mean, 1.58 ± 0.10) than that of sera from dogs with CVL against LRP (mean, 0.64 ± 0.28). The reactivity of the healthy sera was also higher against SLA (mean, 0.41 ± 0.1) than against LRP (mean, 0.09 ± 0.04). The cutoff values (defined as the mean reactivity value from healthy sera plus 3 standard deviations) were 0.213 for LRPs and 0.735 for SLA. These cutoff values allowed us to identify positive and negative sera and, consequently, to estimate the performance parameters of the ELISA for the diagnosis of symptomatic CVL (Table 1). When the sera from oligosymptomatic and asymptomatic dogs were included in the analysis, ELISA with LRPs gave the best results in terms of sensitivity: 100% for both groups ($P < 0.001$; the mean of the absorbance values observed for sera from oligosymptomatic and asymptomatic dogs compared with the mean of the absorbance value for sera from healthy dogs) (Fig. 2). On the other hand, the SLA-based ELISA was only 59% sensitive (10/17) with sera from oligosymptomatic dogs ($P < 0.01$; the mean of the absorbance values observed for sera from oligosymptomatic dogs compared with the mean of the absorbance value for sera from healthy dogs). In addition, the SLA-based ELISA detected considerably fewer asymptomatic cases (19%; 4/22) than the LRP-based ELISA, with no statistically significant differences in the mean of the absorbance values for these sera and the mean of the absorbance for sera from healthy dogs being observed (Fig. 2).

**Cross-reactivity of LRPs and SLA.** Since LRP is composed of evolutive conserved proteins in *Leishmania*, we have analyzed the potential cross-reactions of the LRP extracts with the sera from dogs infected with other unicellular protozoa: *Toxoplasma gondii* ($n = 5$) and *Trypanosoma cruzi* ($n = 9$). In Fig.

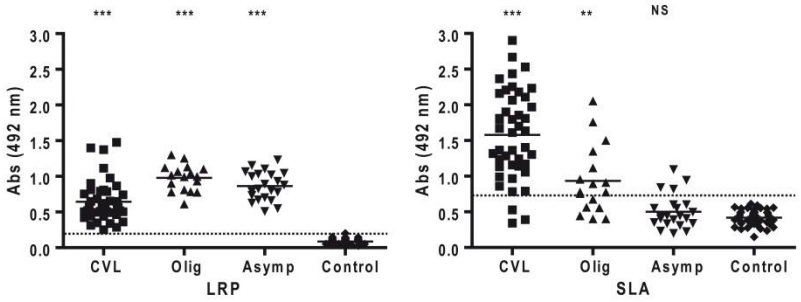


FIG. 2. Comparative evaluation of the diagnostic sensitivities of the *Leishmania infantum* LRPs and the *Leishmania chagasi* SLA. The reactivities of sera collected from dogs with symptomatic, oligosymptomatic, and asymptomatic CVL against LRPs and SLA were analyzed by ELISA. As a control, the reactivities of sera from healthy (uninfected) dogs living in regions where VL is endemic against LRPs and SLA were measured. Solid lines, mean values of reactivity for different groups of sera; dotted lines, cutoff value, defined as the mean optical density plus 3 standard deviations of the values obtained with sera from healthy controls. Abs, absorbance; Olig, oligosymptomatic CVL; Asymp, asymptomatic CVL; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant ($P \geq 0.05$).

3A, the reactivity values of the individual sera for each group against LRPs are shown. None of the sera from the *T. gondii*-infected dogs (mean, 0.1012 ± 0.056) or the *T. cruzi*-infected dogs (mean, 0.101 ± 0.06) showed reactivity above the cutoff defined by the sera from healthy dogs. When the reactivities of these two groups of sera to LRPs were compared with the responses shown by the sera from the healthy dogs, there were no statistically significant differences (Fig. 3A). On the other hand, the mean reactivities of these sera against SLA (0.629 ± 0.21 for *T. gondii*-infected dogs and 0.99 ± 0.29 for *T. cruzi*-infected dogs) were higher than those observed against LRPs, with some of the absorbance values being above the cutoff (two of five serum samples for *T. gondii*-infected dogs and eight of nine serum samples for *T. cruzi*-infected dogs). Yet the reactivity against SLA of the sera from the *T. cruzi*-infected dogs was statistically significantly different ($P < 0.05$) compared with the reactivity against SLA of the sera from healthy dogs (Fig. 3A).

Finally, the reactivities of the sera from dogs vaccinated with two *Leishmania* prophylactic vaccines licensed in Brazil, Leishmune (39) and Leishtec (19), against the LRP and SLA extracts were assayed. Although there were no statistically significant differences between the sera from Leishmune- or Leishtec-vaccinated dogs and the sera from the healthy control dogs, we found that 22.2% (4/18) of the serum samples from dogs vaccinated with Leishmune showed absorbance values above the cutoff when the LRP extracts were used in the ELISAs (Fig. 3B). When the sera from the Leishmune-vaccinated dogs were analyzed in the SLA-based ELISAs, the same percentage (22.2%; 4/18) showed reactivity values above the cutoff, with 3 of these 4 serum samples being the same samples that showed cross-reactivity with LRPs. None of the 23 serum samples obtained from dogs vaccinated with Leishtec showed reactivity against SLA. Only one of these serum samples showed reactivity against LRPs and had an optical density value close to the cutoff defined by the negative sera from the healthy controls. When the reactivities of these two groups of serum samples to LRPs and SLA were compared, the mean absorbance values were higher for the SLA extracts (0.52 ± 0.24 for Leishmune and 0.19 ± 0.1 for Leishtec) than for the LRP extracts (0.24 ± 0.13 for Leishmune and 0.08 ± 0.04 for Leishtec).

TABLE 1. Sensitivities and specificities of ELISAs with LRPs and SLA for serodiagnosis of symptomatic dogs infected with *L. chagasi*

| Antigen | Sensitivity[a] (%) | Specificity[b] (%) | PPV[c] (%) | NPV[d] (%) |
|---|---|---|---|---|
| LRP | 100 (0/44) | 98.2 (1/47) | 97.70 | 100 |
| SLA | 96 (3/44) | 100 (0/47) | 100 | 94 |

[a] Sensitivity was calculated from the equation [number of true-positive samples/(number of true-positive samples + number of false-negative samples)] × 100. The number of samples with false-negative results/total number of samples tested is indicated in parentheses.
[b] Specificity was calculated from the equation [number of true-negative samples /(number of true-negative samples + number of false-positive samples)] × 100. The number of samples with false-positive results/total number of samples tested is indicated in parentheses.
[c] PPV, positive predictive value, which was calculated from the equation [number of true-positive samples/(number of true-positive samples + number of false-positive samples)] × 100.
[d] NPV, negative predictive value, which was calculated from the equation [number of true-negative samples/(number of true-negative samples + number of false-negative samples)] × 100.

## DISCUSSION

As occurs with other *Leishmania* intracellular proteins, such as histones, cysteine proteinases, or kinesine (3, 14, 15, 41, 43, 46), we show in this work that during CVL many of the ribosome constituents are antigenic proteins. Since the antigenicity of the parasite ribosomal proteins was also demonstrated in mouse models of cutaneous leishmaniasis (28), it can be deduced that parasite ribosomes interact with the immune system of the vertebrate hosts during natural and experimental *Leishmania* infections. Parasite ribosomal proteins were employed as the source of antigen for ELISA, a precise and sensitive technique for the screening of a large number of samples for the diagnosis of VL (21, 44). A comparative analysis of the LRP extracts and total parasite proteins obtained from promastigote lysates was performed because the use of crude

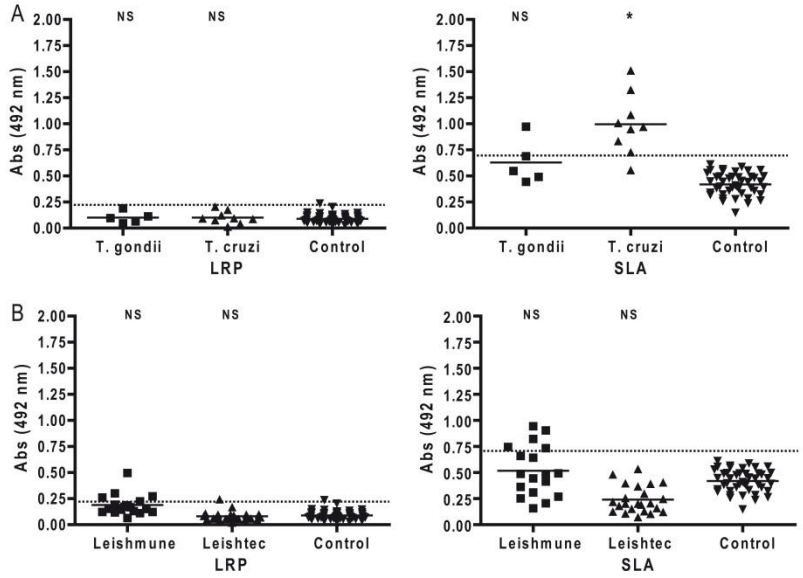


FIG. 3. Comparative evaluation of the diagnostic specificity of *Leishmania infantum* LRPs and *Leishmania chagasi* SLA. (A) ELISA reactivities of sera from dogs infected with *T. gondii* or *T. cruzi* with LRPs and SLA. (B) ELISA reactivities of sera from dogs vaccinated with the Leishmune or the Leishtec vaccine with LRPs and SLA. As a control, the reactivities of sera from healthy (uninfected) dogs living in regions where VL is endemic against LRPs and SLA were measured. Solid lines, mean value of the reactivity; dotted lines, cutoff value, defined as the mean optical density plus 3 standard deviations of the values obtained with sera from healthy controls. Abs, absorbance; *, $P < 0.05$; NS, not significant ($P \geq 0.05$).

SLA-based ELISAs has usually provided a high degree of sensitivity for the diagnosis of VL (6, 31, 44).

By using sera from dogs with symptomatic CVL in a region of Brazil where VL is endemic, the sensitivity and specificity values obtained with the LRP extracts were similar to those shown by the use of SLA when the sera from healthy dogs obtained in the same region were employed as a control. A slight increase in sensitivity was obtained with LRP compared with that achieved with SLA (100% and 96%, respectively), and only one of the serum samples obtained from healthy dogs showed an absorbance value against LRP above the cutoff defined by the reactivity of the control sera. In addition, 100% of the serum samples from oligosymptomatic dogs assayed had a positive reaction against LRP, but only 59% were found to be positive when SLA was used. Thus, it can be concluded that the diagnostic performance of the LRP-based ELISA was similar to that of the SLA-based ELISA for the diagnosis of symptomatic CVL. In addition, the sensitivity of the LRP-based ELISA was higher than that of the SLA-based ELISA for the diagnosis of oligosymptomatic CVL.

Serodiagnosis is usually carried out to confirm the presence of CVL in dogs presenting with clinical manifestations (4, 26). However, the detection of asymptomatic dogs may be critical in epidemiological studies for controlling the spread of the disease among dogs and also between dogs and humans, since seropositive asymptomatic dogs have been implicated in the transmission of the parasite to the insect vector (18, 33, 36). Since the SLA-based ELISA failed to detect a large percentage of asymptomatic cases of CVL (34, 42), we analyzed the sensitivity of the LRP extracts for the diagnosis of asymptomatic CVL. Whereas the LRP antigen mixture detected all the asymptomatic cases (100%), the assay with the SLA preparation detected only about 19% of the cases. Although the reactivity against LRP needs further confirmation by use of a larger number of serum samples from asymptomatic dogs, the use of LRP-based ELISAs in combination with other proposed parasitological, cellular, and serological tests may be useful for the detection of asymptomatic dogs in areas of endemicity (11, 38). In addition, we are analyzing the correlation between seroreactivity against LRP and the transmission of the parasites to the insect vector, as well as the clinical evolution of LRP-positive asymptomatic dogs.

The specificity of the ELISA with SLA largely depends on the antigen preparation, and some false-positive results were obtained with the sera collected from patients or dogs with diseases that are coendemic with VL or CVL, such as Chagas' disease, malaria, leprosy, and toxoplasmosis (21, 29, 42). For this reason, several parasitic recombinant proteins have been individually employed as the antigen in ELISAs for the development of a more specific diagnostic test (30). Comparative ELISAs generally revealed higher specificities but lower sensitivities when individual recombinant antigens instead of SLA were employed for the diagnosis of human VL (31) or CVL (42). Lower sensitivity values were related to the variability in the heterogeneous humoral response elicited against parasite proteins observed in each patient or infected dog. The use of a combination of nonrelated antigens (25, 42) or the production of polyproteins containing several parasite antigens (7, 49)

could further improve the performance of the ELISAs. Alternatively, purified parasite fractions containing different parasite antigens have been shown to be an alternative for the development of sensitive and specific tests for the diagnosis of CVL (6). Since the sera from *T. cruzi*- or *T. gondii*-infected dogs were not able to recognize LRP extracts, whereas some of these sera showed a high degree of reactivity against SLA, our data indicate that LRP can be employed as a more specific antigen than SLA for the differential diagnosis of CVL.

In order to define the composition of future diagnostic tests based on parasite ribosomal proteins, we are now characterizing the main antigenic components of the parasite ribosomes. The use of these molecules in combination with other parasite recombinant antigens recognized by sera from different percentages of symptomatic and asymptomatic dogs with CVL, such as rK39 (25), A2 (13), and tryparedoxin peroxidase (47), may promote the development of more specific and sensitive tests for the diagnosis of CVL.

The diagnostic specificity of the test should also be maintained when sera are obtained from vaccinated dogs. Due to the existence of licensed commercial vaccines in Brazil (19, 39), it would be desirable to differentiate infected dogs from vaccinated animals. Our results show that while some of the serum samples from animals vaccinated with Leishmune showed some reactivity against LRPs and SLA, the sera from none of the animals vaccinated with Leishtec reacted against these antigenic preparations. Palatnik-de-Sousa et al. (40) have described the protective effect of the fucose-mannose ligand vaccine, like the Leishmune vaccine, against CVL. As expected with the use of saponin adjuvants, the Leishmune vaccine induces a strong humoral immune response soon after the complete vaccination is provided (9). Therefore, an indistinct IgG-mediated anti-*L. chagasi* immune response is detected in Leishmune-vaccinated dogs, and that response is indistinct from that due to natural infection with *L. chagasi*. This fact can explain the reactivity of some serum samples obtained when *Leishmania* antigens (LRPs and SLA) were used in the ELISAs.

Taken together, the results presented here demonstrate that the LRP extracts may be considered an interesting alternative for use for the diagnosis of CVL by ELISA, mainly in asymptomatic animals, in epidemiological studies in areas of endemicity.

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**Resumen del artículo: "BALB/c mice vaccinated with *Leishmania major* ribosomal proteins extracts combined with CpG oligodeoxynucleotides become resistant to disease caused by a secondary parasite challenge". Journal of Biomedicine and Biotechnology (JBB), 2010. Art. Id. 181690.**

Existe un antecedente bibliográfico al trabajo presentado en esta Tesis Doctoral. En el año 2008, Iborra y colaboradores (Referencia número 15 del artículo aquí resumido; JBB) demostraron que la inmunización de extractos ribosómicos de *Leishmania* (LRP) en combinación con un adyuvante inductor de respuestas Th1 (CpG-ODN) en ratones BALB/c y C57BL/6 era capaz de proteger a los animales del desarrollo de la LC causada por la infección por *L. major*. En el artículo que aquí se presenta se planteó estudiar en mayor profundidad la protección inducida por la vacuna en el modelo susceptible de LC (*L. major*-BALB/c), además de analizar si estos animales serían capaces de resistir a una segunda infección.

En primer lugar se alargó el tiempo de estudio tras la infección. Los excelentes parámetros clínicos, parasitológicos e inmunes obtenidos más de cuatro meses tras la infección en estos animales (Figura 1; JBB) avalaron la hipótesis de analizar una segunda infección. Tras ésta, los resultados obtenidos mostraron que los ratones vacunados y reinfectados eran resistentes a la infección secundaria (Figura 2A y B; JBB). Esta resistencia se asoció tanto a la inducción de respuestas mediadas por IFN-γ como al control de las respuestas asociadas con la patología (mediadas por IL-4 e IL-10) (Figuras 3 y 4; JBB). Se discute en el trabajo el posible papel de la cronificación de la infección primaria (puesta de manifiesto por la presencia del parásito en los ganglios que drenan el sitio de infección primaria, [Figura 2C; JBB]) y la resistencia a la reinfección. Los datos que se muestran en este trabajo apoyan el hecho de que los ribosomas son partículas capaces de producir una fuerte protección y refuerzan al ribosoma como un candidato idóneo para el desarrollo de vacunas contra las leishmaniosis causadas por *L. major*.

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*Research Article*

# BALB/c Mice Vaccinated with *Leishmania major* Ribosomal Proteins Extracts Combined with CpG Oligodeoxynucleotides Become Resistant to Disease Caused by a Secondary Parasite Challenge

**Laura Ramírez,[1] Salvador Iborra,[2] Jimena Cortés,[1] Pedro Bonay,[1] Carlos Alonso,[1] Manoel Barral-Netto,[3] and Manuel Soto[1]**

[1] *Departamento de Biología Molecular, Centro de Biología Molecular Severo Ochoa, (CSIC-UAM), Nicolás Cabrera 1, Universidad Autónoma de Madrid, 28049 Madrid, Spain*
[2] *Unidad de Inmunología Viral, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Crta. Pozuelo Km 2, 28220 Majadahonda, Madrid, Spain*
[3] *Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz (FIOCRUZ), Rua Waldemar Falcao, 121, Candeal, 40.296-710 Salvador-Bahia, Brazil*

Correspondence should be addressed to Manuel Soto, msoto@cbm.uam.es

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Leishmaniasis is an increasing public health problem and effective vaccines are not currently available. We have previously demonstrated that vaccination with ribosomal proteins extracts administered in combination of CpG oligodeoxynucleotides protects susceptible BALB/c mice against primary *Leishmania major* infection. Here, we evaluate the long-term immunity to secondary infection conferred by this vaccine. We show that vaccinated and infected BALB/c mice were able to control a secondary *Leishmania major* challenge, since no inflammation and very low number of parasites were observed in the site of reinfection. In addition, although an increment in the parasite burden was observed in the draining lymph nodes of the primary site of infection we did not detected inflammatory lesions at that site. Resistance against reinfection correlated to a predominant Th1 response against parasite antigens. Thus, cell cultures established from spleens and the draining lymph node of the secondary site of infection produced high levels of parasite specific IFN-$\gamma$ in the absence of IL-4 and IL-10 cytokine production. In addition, reinfected mice showed a high IgG2a/IgG1 ratio for anti-*Leishmania* antibodies. Our results suggest that ribosomal vaccine, which prevents pathology in a primary challenge, in combination with parasite persistence might be effective for long term maintenance of immunity.

## 1. Introduction

Protozoa of the genus *Leishmania* are obligate intracellular parasites of the mononuclear phagocytic lineage. *Leishmania* infection causes a group of diseases ranging from self-healing cutaneous ulcers to potentially lethal fatal visceral infection, globally known as leishmaniasis [1]. *L. major* is the main causative agent of cutaneous leishmaniasis (CL) in the Old World. In humans, CL due to *L. major* infection is self-limiting and healing is associated with resistance to reinfection. This acquired immunity to reinfection in natural *Leishmania* hosts suggests that a vaccine is feasible. However, there are no available vaccines against human leishmaniasis [2].

Effective primary immunity against *L. major* in mouse requires an IL-12 dependent production of IFN-$\gamma$ from $CD4^+$T cells (Th1 response) and $CD8^+$ T cells that mediates a nitric oxide-dependent killing by infected macrophages [3, 4]. In contrast, susceptibility correlates with the dominance of an IL-4 driven Th2 response, as it has been observed in certain mice strains like BALB/c [3, 4]. Subcutaneous (s.c.) experimental infection of BALB/c mice with a high

dose inoculum of stationary phase promastigotes induces rapidly evolving lesions that correlated with the generation of strong Th2 responses [5]. This model of experimental CL has been extensively used to explore the protective role of several parasite antigens combined with different adjuvants [2, 6, 7]. The immunization with certain parasite proteins, irrespective of their cellular location (surface or intracellular parasite antigens), inoculated with Th1 modulating adjuvants can induce immune responses that resulted in protection [8, 9]. The production of parasite specific IFN-$\gamma$ combined with the control of the production of the disease associated IL-4 and IL-10 cytokines has been correlated to protection against the development of CL in vaccinated BALB/c mice [10]. Protective cell mediated immunity can also be induced in BALB/c mice after s.c. infection using a nonpathogenic challenge of *L. major* promastigotes (leishmanization) [11–14]. Leishmanized mice developed very low or no pathology after primary infection and acquired resistance against a pathogenic rechallenge [11, 13, 14]. Leishmanization induced parasite specific Th1 responses that were able to control the secondary challenge made in a distant site [13, 14].

In a previous work, we have shown that during *L. major* infection, susceptible BALB/c mice develop a Th2 response against parasite ribosomal crude extracts purified from promastigotes [15]. Vaccination with the parasite ribosomal proteins (LRP) combined with CpG oligodeoxynucleotides (CpG ODN) as adjuvant induced a specific Th1 response, since vaccinated mice developed anti-LRP antibodies of the IgG2a isotype and their splenocytes produced high amounts of IFN-$\gamma$, but not IL-4, after in vitro stimulation with LRP [15]. The immune state induced by vaccination conferred protection against a primary challenge with *L. major* parasites in the footpad. After infection, a *Leishmania* specific IL-12 dependent production of IFN-$\gamma$ and a reduced production of IL-4 and IL-10 were associated to protection [15].

In this work, we have analyzed whether or not vaccinated and protected mice were able to control the development of CL after a secondary challenge. To this end, mice vaccinated with LRP + CpG ODN were infected in the footpad with a pathogenic challenge of *L. major* parasites. The development of footpad swelling was analyzed over a period of 18 weeks as a stringent test of vaccine induced protection. Since no CL pathology was found during the follow up, mice were reinfected into the ear dermis with a low dose pathogenic challenge of *L. major* metacyclic promastigotes. Our results showed that vaccinated and infected mice developed a resistant phenotype to parasite associated disease at a secondary site of infection.

## 2. Materials and Methods

*2.1. Animals and Parasites.* Female BALB/c mice (4–6 week-old) were purchased from Harlan Interfauna Ibérica S.A. (Barcelona, Spain). *L. major* parasites (WHOM/IR/-/173) and clone V1 (MHOM/IL/80(Friedlin)) were kept in a virulent state by passage in BALB/c mice. *L. major* amastigotes were obtained and transformed to promastigote by culturing at 26°C in Schneider's medium (Gibco, BRL, Grand Island, NY, USA) supplemented with 20% foetal calf serum. Metacyclic promastigotes of *L. major* (clone V1) were isolated from stationary cultures by negative selection as described in [16] using peanut agglutinin (Vector Laboratories, Burlingame, CA, USA).

*2.2. Parasite Antigens, Adjuvant and Immunizations .* Soluble *Leishmania major* antigen (SLA) was prepared as described [17]. Briefly, *L. major* promastigotes were harvested from culture and washed four times in phosphate-buffered saline (PBS). The parasites were suspended in PBS and subjected to three freezing and thawing cycles and sonicated with five cycles of 30 seconds at 38 MHz. After cell lysis, soluble antigens were separated from the insoluble fraction by centrifugation for 15 minutes at 12,000 $\times$ g using a microcentrifuge. *L. major* ribosomal proteins (LRP) were prepared as described [15]. Phosphorothioate-modified CpG ODN (5′-TCAACGTTGA-3′ and 5′- GCTAGCGTTAGCGT-3′) were synthesized by Isogen (The Netherlands).

Six mice were s.c. immunized in the right footpad with 12 $\mu$g of *L. major* LRP combined with 25 $\mu$g of each CpG ODN in a volume of 30 $\mu$l. Control groups ($n = 6$) received either CpG ODN or phosphate saline buffer PBS. Mice were immunized three times at two-week intervals.

*2.3. Parasite Challenge.* The primary parasite challenge was done by s.c. inoculation in the left footpad with $5 \times 10^4$ stationary-phase promastigotes of *L. major* (WHOM/IR/-/173) in a volume of 30 $\mu$l, four weeks after the last vaccine inoculation. The secondary infection was done at week 18 after primary infection with 1000 metacyclic promastigote of *L. major* (clone V1) isolated from stationary cultures by negative selection using peanut agglutinin (Vector Laboratories, Burlingame, CA). Metacyclic forms were injected into the dermis (i.d.) of both ears of each mouse in a volume of 10 $\mu$l.

Footpad swelling was measured with a metric calliper and calculated as thickness of the left footpad minus thickness of the right footpad. Evolution of the ear lesion was monitored by measuring the diameter of the indurations with a metric calliper.

*2.4. Estimation of Parasitic Load.* The number of parasites was determined by limiting dilution assay [18]. Briefly, ears were recovered from infected mice and the ventral and dorsal sheets were separated. Ear sheets were deposited in RPMI medium containing Liberase CI enzyme blend (50 $\mu$g ml$^{-1}$) (Roche, Mannheim, Germany). After an incubation period of 2 hours at 37°C, the tissues were cut into small pieces, homogenized and filtered using a cell strainer (70 $\mu$m-pore size). The homogenized tissue was serially diluted in a 96-well flat-bottomed microtiter plate containing Schneider's medium plus 20% FCS. The number of viable parasites was determined from the highest dilution at which promastigotes could be grown up to 7-day incubation at 26°C. The number of parasites was also determined in the local draining

lymph nodes (DLN) of infected ears (retromaxillar) and footpad (popliteal) and in the spleen. Organs were recovered, mechanically dissociated, homogenized and filtered and then serially diluted as above. Parasite load is expressed as the number of parasites in the whole organ.

*2.5. Measurement of Cytokines in Supernatants.* Splenocytes and DLN cells suspensions were seeded in complete RPMI medium (RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, and 10 mM 2-mercaptoethanol). $3 \times 10^6$ cells were seeded in 48-well plates during 48-hour at 37°C in the presence of LRP ($12\,\mu g\,ml^{-1}$) or SLA ($12\,\mu g\,ml^{-1}$). The release of IFN-$\gamma$, IL-10, and IL-4 was measured in the supernatants of splenocytes and DLN cell cultures using commercial ELISA kits (Diaclone, Besançon, France).

*2.6. Analysis of the Humoral Responses.* Reciprocal end-point titre (defined as the inverse of the highest serum dilution factor giving an absorbance >0.2) against LRP and SLA was determined by serial dilution of the sera assayed by ELISA using anti-IgG1 (1/1000) and anti-IgG2a (1/500) horseradish peroxidase-conjugated anti-mouse immunoglobulins as secondary antibodies (Nordic Immunological Laboratories, Tilburg, The Netherlands). Plates were coated with 100 $\mu$l of LRP ($5\,\mu g\,ml^{-1}$ in PBS) or SLA ($2\,\mu g\,ml^{-1}$ in PBS).

*2.7. Statistical Analysis.* Statistical analysis was performed by a Student's $t$-test. Differences were considered significant when $P < .05$.

## 3. Results and Discussion

*3.1. Protective Immunity Generated by s.c. Vaccination with LRP + CpG ODN in the Footpad.* In a previous work we showed that BALB/c mice vaccinated with LRP combined with CpG ODN were protected against the development of cutaneous lesions in the footpad 8 weeks after parasite challenge [15]. The absence of footpad swelling was correlated with a 3-log reduction in parasite burden in the ipsilateral popliteal DLN when compared with mice immunized with the adjuvant or the excipient (control groups) [15]. In addition, no parasites were found in the spleen of the LRP + CpG ODN vaccinated animals whereas control groups contained approximately $10^4$ parasites. It was concluded that the Th1 immune response induced in BALB/c mice by the vaccination of the LRP combined with CpG ODN resulted in a solid immunity that efficiently controlled parasite induced cutaneous disease maintaining a chronic infection in the local DLN [15]. In this work, we decided to analyze the footpad swelling of LRP + CpG ODN vaccinated mice after a longer period of time. After parasite challenge, vaccinated mice did not develop lesion for up to eighteen weeks (Figure 1(a)). Since control groups were sacrificed at week seven after challenge (because they began to develop severe necrotic lesions) a comparative analysis between controls and LRP + CpG ODN vaccinated mice was not possible. However, the parasite burden in the spleen and in the popliteal DLN of the LRP + CpG ODN vaccinated mice was analyzed at week 18 after parasite challenge in the footpad. As it is shown in Figure 1(b), no parasites could be detected in the spleen of the vaccinated mice. The number of parasites located at the popliteal DLN at week 18 after challenge ($5.41 \pm 0.99$; $\log_{10}$ scale) represents a 1.12-log increment ($P = .23$) when compared with the number of parasites detected in the same organ in LRP + CpG ODN vaccinated mice 8 weeks after challenge ($4.84 \pm 0.26$; $\log_{10}$ scale) [15]. Although a slightly increment in the number of parasites was detected, the presence of high levels of IFN-$\gamma$ measured in the supernatants of DLN cells cultures after stimulation with SLA and LRP in the absence of detectable levels of IL-4 and IL-10 (Figure 1(c)), should be taken as an indication that the parasite-specific Th1 response observed at week 8 [15] was maintained at week 18 after challenge. Thus, the Th1 response elicited by LRP + CpG ODN vaccination was able to induce an immunological status that protects mice against the development of cutaneous lesions during the 18 weeks of follow up. In addition, a chronic infection was patent in the vaccinated mice, being the parasites maintained located in the local DLN without dissemination to the internal organs.

This study reinforces that CpG ODN provides protection when used in combination with LRP extracts. Previous studies using this adjuvant in combination with parasite lysates showed a different degree of protection against *L. major* infection in the susceptible BALB/c [19–21] and in the resistant C57BL/6 [19] mice strains. The identification of a protein fraction composed by ribosomal proteins that provide protection against the development of cutaneous leishmaniasis lesions represents a substantial step in defining the protective immunogens within SLA, helping to identify new protective parasite antigens for the development of molecularly defined vaccines against leishmaniasis.

*3.2. Protected Mice Became Resistant to Disease Caused by a L. major Rechallenge in the Ear Dermis.* Next, we analyzed if protected mice were able to control a second parasite challenge. For that purpose, vaccinated and protected mice ($n = 6$) were rechallenged in the ear dermis with 1000 *L. major* metacyclic promastigotes, parasite infective forms that seem to be similar to the promastigotes that are inoculated during the insect vector blood feeding [22]. Since vaccines were inoculated in the contralateral footpad of the primary infection site, rechallenge was made by i.d. infection in the ears. BALB/c mice infected with a low dose of *L. major* metacyclic promastigotes develop progressive inflammatory lesion in the spot of infection that increased in size, accompanied by ulceration and tissue necrosis [23–26] as occurred in mice challenged in the footpad with a high dose inoculum of stationary promastigotes [3, 4]. As control six naïve BALB/c mice were also infected in the ear dermis with the same dose of parasites.

Very low dermal lesion development was observed in reinfected mice (Figure 2(a)). In some cases (in four of six mice) a complete absence of inflammation in the ears was observed for up to seven weeks. Two mice developed low dermal lesions (<1 mm) that reached a peak at week 5 and were almost completely healed at week seven. On

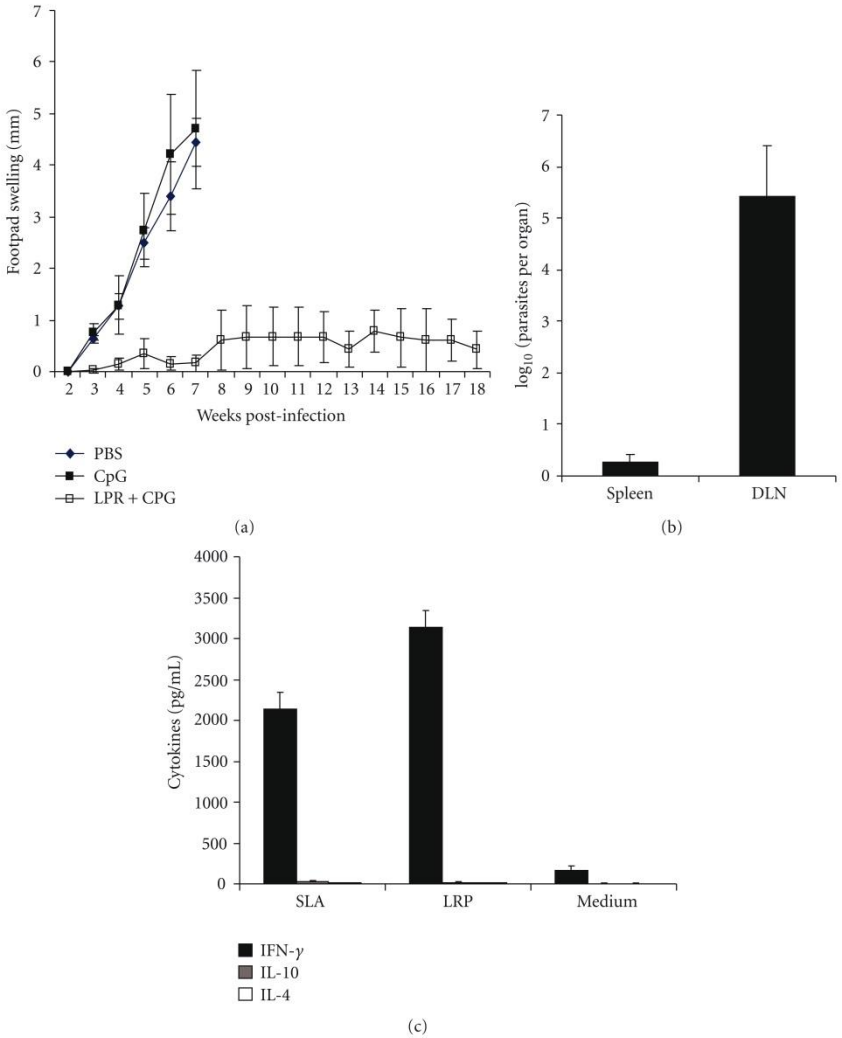


Figure 1: Course of *L. major* infection in BALB/c vaccinated mice. Mice (six per group) were s.c. immunized in the right footpad with three doses of the LRP adjuvated with CpG ODN (LRP + CpG), with the CpG ODN adjuvant (CpG) or with PBS. One month after the last immunization, the animals were infected in the left hind footpad with $5 \times 10^4$ *L. major* stationary phase promastigotes. (a) Footpad swelling is given as the difference of thickness between the infected and the uninfected contralateral footpad. (b) The number of viable parasites in the spleen and the popliteal DLN of the LRP + CpG ODN vaccinated were individually determined by limiting dilution at week eighteen post challenge. Results are expressed as the mean±SD of six spleens and popliteal DLN. (c) At week eighteen after footpad infection the level of IFN-$\gamma$, IL-10 and IL-4 was measure by ELISA in the supernatants of popliteal lymph node cells cultures from LRP + CpG ODN vaccinated mice. Cells were in vitro stimulated for 48 hours with 12 $\mu$g/ml of SLA or LRP and medium alone. Results are expressed as the mean±SD.

the other hand, infection in all control naïve mice leads to the development of progressive inflammatory lesions in the ears (Figure 2(a)). The parasite load in the ear dermis and retromaxillar DLN was analyzed at week seven after challenge. Vaccinated and reinfected mice showed very low parasite loads in the ears and in the retromaxillar DLN, correlating to the absence of parasite in the spleens (Figure 2(b)). These data contrast with the parasite burdens found in the ear dermis and in the retromaxillar DLN in the control group mice. Also, as an indication of parasite dissemination, parasites were detected in the spleen of the control mice (Figure 2(b)). These data indicate that the immune state generated after the first infection in the LRP + CpG ODN vaccinated mice is extremely potent, leading

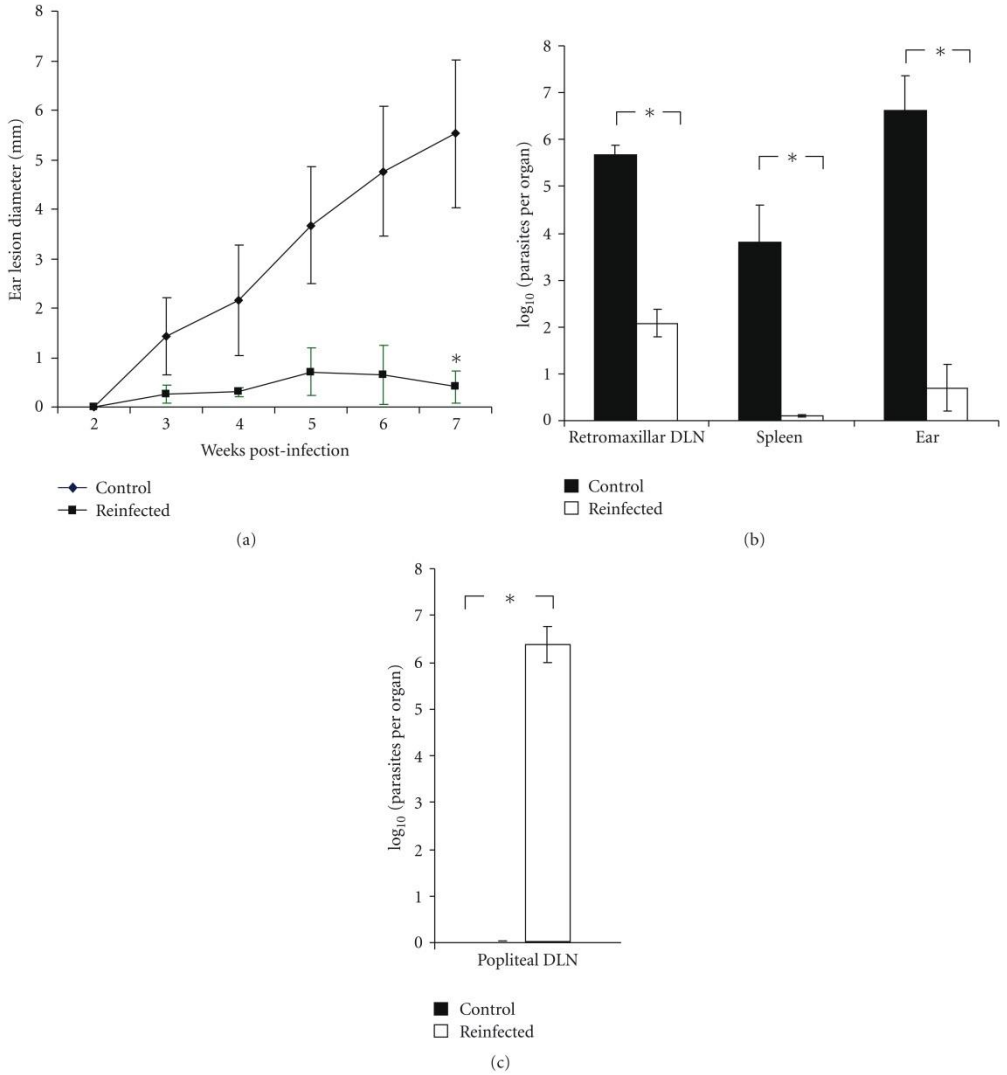


FIGURE 2: (a) Course of *L. major* infection in protected and reinfected BALB/c mice. Values represent the mean lesion diameter±standard deviation (SD). $^*P < .001$: significant differences in inflammation for protected versus control mice at week seven postchallenge. (b) Seven weeks after reinfection, mice were euthanized and parasite burden in the ear dermis, spleen and in the retromaxillar DLN was individually quantitated. Results are expressed as the mean±SD of twelve ears and six spleens and DLN. $^*P < .001$: significant decrease for reinfected versus control mice. (c) The parasite burden at week seven after rechallenge was individually quantitated in the popliteal lymph nodes of control and reinfected mice. Results are expressed as the mean±SD of six DLN. $^*P < .001$: significant decrease for reinfected versus control mice.

to a rapid and efficient control of parasite growth in the site of reinfection, that resulted in the generation of a moderate dermal pathology.

In the vaccinated reinfected mice the primary challenge site was also analyzed, since in immune genetically resistant mice an *L. major* secondary challenge can cause disease reactivation in the primary site despite efficient parasite clearance in the site of reinfection [27, 28]. The parasite load found in the popliteal DLN of the reinfected mice (6.68 ± 0.63; $\log_{10}$ scale) (Figure 2(c)) represents an increment of 1.23-log ($P = .028$) when compared with the number of parasites detected at the moment of the secondary challenge. Since no parasites were found in the popliteal DLN of control mice (Figure 2(c)), parasite dissemination from the ear to

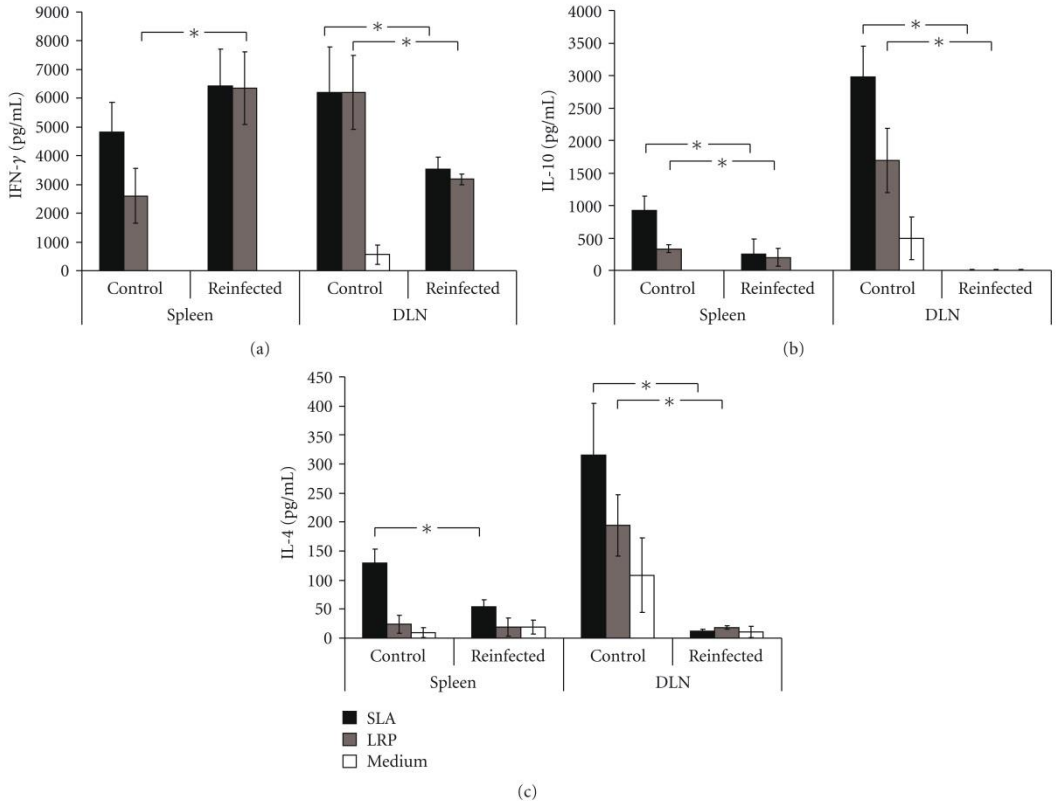


FIGURE 3: Analysis of the cellular responses. At week seven after ear infection the level of IFN-$\gamma$ (a), IL-10 (b), and IL-4 (c) was measured by ELISA in the supernatants of spleen and retromaxillar lymph node cells cultures from both mice groups. Cells were in vitro stimulated for 48 hours with 12 $\mu$g/ml of SLA or LRP and medium alone. Results are expressed as the mean±SD of twelve ears and DLN. ($^*P < .001$).

these lymph nodes seems to be unlikely and the increase in the number of parasites found in the popliteal DLN may be indicating that the secondary challenge induced some parasite replication. However, we did not detect an increment in the footpad swelling in the vaccinated reinfected BALB/c mice for up to seven weeks after secondary challenge (not shown). Thus, we conclude that secondary challenge in the ear dermis did not produce a disease reactivation in these vaccinated mice.

*3.3. Analysis of the Cellular Immune Response.* To determine which immunological parameters are related to resistance after the secondary challenge, the SLA and the LRP driven production of IL-4, IL-10, and IFN-$\gamma$ was assayed at week seven after ear infection. Spleen cell cultures from control and reinfected mice were established to analyze the systemic response and DLN cells (retromaxillar) were cultured to analyze the local response induced by the ear infection. Spleen cells from reinfected mice produced higher amounts of IFN-$\gamma$ after SLA or LRP stimulation than control mice, but only the level of LRP specific IFN-$\gamma$ was significantly different between the two groups. We observed that the level of SLA- and LRP-specific IFN-$\gamma$ detected in the DLN cell cultures was higher in control than in the reinfected mice (Figure 3(a)). Most likely, the high level of IFN-$\gamma$ detected in retromaxillar DLN could be related to the high number of parasites found in control animals (Figure 2(b)) that may be stimulating the production of IFN-$\gamma$ by Th1 cells, since in this model of infection the presence of parasites is correlated with IFN-$\gamma$ production [26]. The IL-10 and IL-4 production after stimulation with SLA or LRP was barely detected in the spleen and DLN cells from reinfected mice whereas in the spleen cell cultures and especially in the DLN cell culture supernatants from control mice high levels of these cytokines were measured (Figures 3(b) and 3(c)).

Those data are compatible with the fact that Th1/Th2 mixed responses were elicited after infection in control mice, characterized by the production of parasite specific IFN-$\gamma$ and IL-4 cytokines. In addition, the presence of high levels of parasite specific IL-10 may be also implicated in the progression of the disease, since the inactivating effect of this cytokine in infected macrophages has been related

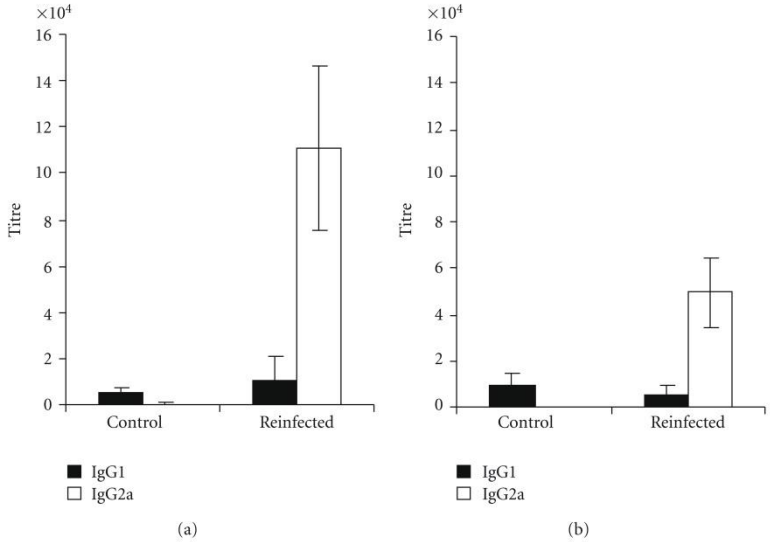


Figure 4: Analysis of the humoral responses. Serum samples from control and vaccinated reinfected mice were obtained seven weeks after challenge in the ear dermis. The titre for IgG1 and IgG2a antibodies against LRP (a) and SLA (b) was determined individually by ELISA. Results are expressed as the mean±SD.

Table 1: Cytokine production by popliteal DLN cells from vaccinated reinfected mice at week seven after secondary challenge.

| | SLA | LRP | Medium |
|---|---|---|---|
| IFN-$\gamma$ | 5837.16 ± 834.82 | 5773.52 ± 1411, 14 | 1635, 11 ± 607, 89 |
| IL-10 | 526.31 ± 214.98 | 408, 92 ± 233.36 | 104, 39 ± 57, 13 |
| IL-4 | 229.54 ± 58.78 | 44.36 ± 31.22 | 54.38 ± 37.89 |

The level of cytokines was determined by ELISA in the supernatant of popliteal DLN cells obtained from reinfected mice at week seven post rechallenge, after in vitro stimulation with 12 $\mu$g/ml of SLA and LRP. Mean±SD of samples from six mice is shown (pg/ml).

with BALB/c mice susceptibility against *L. major* infection [29–32]. The pattern of cytokine production observed in infected control mice, with detectable level of parasite specific production of IFN-$\gamma$, IL-10 and IL-4 was previously observed after infection with a pathogenic challenge of *L. major* in BALB/c ears [25, 26, 33]. On the contrary, a Th1-mediated IFN-$\gamma$ production was elicited in the reinfected mice group in the absence of Th2 responses and IL-10 mediated regulatory responses.

The SLA and the LRP driven production of IL-4, IL-10, and IFN-$\gamma$ was also assayed in the popliteal DLN of the reinfected mice. Although detectable levels of the three cytokines were observed, the level of IFN-$\gamma$ was higher than the levels of IL-10 and IL-4 (Table 1). A high ratio of IFN-$\gamma$/IL-10 and IFN-$\gamma$/IL-4 for both parasite proteins preparations (11.1 and 25.5 for SLA; 14.1 and 130.15 for LRP, respectively) was obtained, indicating that a parasite-specific IFN-$\gamma$ response was still maintained at week seven after secondary challenge in the popliteal DLN, yet in the presence of IL-4 and IL-10 cytokines. This Th1/Th2 mixed response may account for the increment observed in the number of parasites after secondary infection in the popliteal DLN.

*3.4. Analysis of the Humoral Responses.* The humoral response elicited in control mice and in the reinfected mice was analyzed at week seven after parasite challenge in the ear dermis. The titre of anti-LRP and anti-SLA specific IgG1 and IgG2a antibodies were determined, since the presence of IgG1 and IgG2a antibodies is considered a marker of Th2 and Th1 type responses, respectively [34]. In the sera from control mice the anti-*Leishmania* predominant antibodies were of the IgG1 isotype and very low but detectable levels of IgG2a were observed (Figures 4(a) and 4(b)). On the contrary, vaccinated reinfected mice showed high titres of IgG2a antibodies against LRP (Figure 4(a)) and SLA (Figure 4(b)). These humoral responses are in agreement with the nature of cellular responses observed after in vitro stimulation with both antigenic preparations. A strong Th1 response was elicited in vaccinated reinfected mice after parasite rechallenge having a resistant phenotype. On the contrary, antibodies found in the sera from mice of the control group were mainly of the IgG1 isotype as expected for their nonhealing phenotypes.

Altogether, our data showed that the immune response elicited in the LRP + CpG ODN vaccinated mice after

the primary infection was able to control a secondary challenge. Acquisition of the resistant phenotype was correlated to the capacity to induce a Th1 response (large amounts of parasite specific production of IFN-$\gamma$ and a high anti-leishmanial proteins IgG2a/IgG1 ratio) in the absence of Th2 or IL-10 mediated responses. The immune responses associated with the resistance after secondary infection in the vaccinated-infected mice were similar to that obtained in BALB/c mice that controlled a secondary infection in the ear after a primary infection in the contralateral ear, showing a Th1 response after rechallenge [26]. It is important to note that protection in these mice only occurred when lesions were developed in the primary site of infection [26], whereas LRP + CpG ODN vaccinated mice became resistant after primary challenge without the development of dermal lesions. Also, protection to reinfection achieved by BALB/c mice infected with a low infection dose in the footpad was dependent on the induction of Th1 responses [11, 35]. Here we show that that the immune response elicited by the LRP + CpG ODN vaccine after primary challenge was able to control the development of lesions and generated a long-term immune state necessary for the maintenance of immunity to further infection. The presence of parasites in the popliteal DLN may be related with this Th1 response, since it has been demonstrated that the presence of parasite antigens is necessary for the maintenance of cell mediated immunity in BALB/c mice [14, 36].

## 4. Conclusion

The data reported here provided evidence that BALB/c mice protected against the development of dermal pathology due to *L. major* s.c challenge after LRP + CpG ODN vaccination have acquired an immunological status which conferred them the capacity to resist a further infection (an appealing feature for a vaccine that might be employed in endemic areas, where reexposure to the parasite would be very frequent). After a secondary challenge in the ear dermis, these mice showed a robust protection against *L. major* infection. Very low parasite burdens and development of dermal lesions in the site of reinfection were found. A specific Th1 protective response after the secondary challenge was correlated to resistance to reinfection. Thus, the immune state generated by the combination of vaccination with LRP + CpG ODN and the primary infection is extremely potent, leading to a rapid and efficient elimination of the parasite from the site of reinfection. Despite extensive research efforts, leishmanization with viable *Leishmania* parasites is the only vaccine with proven efficacy in humans [2, 37]. The induction of long-term immune responses by vaccines based on parasite extracts or recombinant parasite products that protected mice against the development of leishmaniosis after a primary challenge has been extensively reported [8, 38]. However, there is scarcity of studies analyzing the long-term maintenance of resistance to reinfection of vaccinated mice. Extrapolation of this approach to other animal or human models is hazardous but our findings may be relevant to develop effective tools against leishmaniasis based on defined *Leishmania* subunits.

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## Resumen del artículo:"Vaccination with the *Leishmania infantum* ribosomal proteins induces protection in BALB/c mice against *Leishmania chagasi* and *Leishmania amazonensis* challenge". Microbes and Infection (M&I), 2010. 12:967-977

Las leishmaniosis son un conjunto de enfermedades provocadas por la infección de parásitos del género *Leishmania*. Los ribosomas son partículas muy conservadas entre las diferentes especies de este parásito, por lo que se hipotetizó que podrían ser unos buenos candidatos para generar protección frente a diferentes especies del género *Leishmania*. En el trabajo aquí resumido se analizó la capacidad protectora de los ribosomas de *Leishmania* frente a la infección por *L. chagasi*, uno de los agentes causales de LV, y *L. amazonensis*, especie del nuevo mundo que provoca una grave LC que en ocasiones puede visceralizar.

Los ratones fueron vacunados con extractos de ribosomas, en presencia de saponina, un adyuvante inductor de respuestas celulares aprobado para uso veterinario. Esta formulación fue capaz de generar de forma específica citoquinas y anticuerpos compatibles con la inducción de respuestas Th1 (Figura 1; M&I). Estas respuestas se correlacionaron con la inducción de protección frente a la infección por ambas especies: la viscerotrópica (Figura 2; M&I) y la cutaneotrópica (Figura 3; M&I). En ambos modelos, la vacunación generó un estado inmunitario capaz de reducir la carga parasitaria aumentando la capacidad leishmanicida de los macrófagos de los animales vacunados (Figura 2AB y Figura 3BC; M&I), reduciendo además las manifestaciones clínicas provocadas por la infección por *L. amazonensis* (Figura 3A; M&I). El estudio de la respuesta inmunológica asociada al control de la enfermedad (Figura 4 y Figura 5; M&I) apoya anteriores observaciones en animales vacunados con LRP e infectados por *L. major*. En estos estudios se relaciona la protección generada por la vacuna con la inducción de respuestas inflamatorias controladas acompañadas por la reducción de respuestas mediadas por IL-10 e IL-4. Los datos del presente trabajo (junto a los presentados en la referencia anterior) permiten presentar a los ribosomas como serios candidatos al desarrollo de vacunas frente a las diferentes formas de las leishmaniosis humanas.

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Original article

# Vaccination with the *Leishmania infantum* ribosomal proteins induces protection in BALB/c mice against *Leishmania chagasi* and *Leishmania amazonensis* challenge

Miguel A. Chávez-Fumagalli [a,1], Mariana A.F. Costa [b,1], Dulcilene M. Oliveira [c,1], Laura Ramírez [d], Lourena E. Costa [e], Mariana C. Duarte [e], Vivian T. Martins [e], Jamil S. Oliveira [b], Carlos C. Olortegi [b], Pedro Bonay [d], Carlos Alonso [d], Carlos A.P. Tavares [b], Manuel Soto [d,*,1], Eduardo A.F. Coelho [b,e,1]

[a] *Departamento de Farmacologia, Universidade Federal de Minas Gerais, 31.270-901, Belo Horizonte, Minas Gerais, Brazil*
[b] *Departamento de Bioquímica e Imunologia, Universidade Federal de Minas Gerais, 31.270-901, Belo Horizonte, Minas Gerais, Brazil*
[c] *Departamento de Neurociências, Universidade Federal de Minas Gerais, 31.270-901, Belo Horizonte, Minas Gerais, Brazil*
[d] *Centro de Biología Molecular Severo Ochoa, CSIC-UAM, Departamento de Biología Molecular, Universidad Autónoma de Madrid, 28049 Madrid, Spain*
[e] *Departamento de Patologia Clínica, Coltec, Universidade Federal de Minas Gerais, 31.270-901, Belo Horizonte, Minas Gerais, Brazil*

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**Abstract**

*Leishmania chagasi* and *Leishmania amazonensis* are the etiologic agents of different clinical forms of human leishmaniasis in South America. In an attempt to select candidate antigens for a vaccine protecting against different *Leishmania* species, the efficacy of vaccination using *Leishmania* ribosomal proteins and saponin as adjuvant was examined in BALB/c mice against challenge infection with both parasite species. Mice vaccinated with parasite ribosomal proteins purified from *Leishmania infantum* plus saponin showed a specific production of IFN-γ, IL-12 and GM-CSF after *in vitro* stimulation with *L. infantum* ribosomal proteins. Vaccinated mice showed a reduction in the liver and spleen parasite burdens after *L. chagasi* infection. After *L. amazonensis* challenge, vaccinated mice showed a decrease of the dermal pathology and a reduction in the parasite loads in the footpad and spleen. In both models, protection was correlated to an IL-12-dependent production of IFN-γ by $CD4^+$ and $CD8^+$ T cells that activate macrophages for the synthesis of NO. In the protected mice a decrease in the parasite-mediated IL-4 and IL-10 responses was also observed. In mice challenged with *L. amazonensis*, lower levels of anti-parasite-specific antibodies were detected. Thus, *Leishmania* ribosomal proteins plus saponin fits the requirements to compose a pan-*Leishmania* vaccine.


*Keywords:* *Leishmania*; Visceral leishmaniasis; Tegumentary leishmaniasis; BALB/c mice; *Leishmania* ribosomal proteins; Vaccine

## 1. Introduction

Leishmaniasis are a group of vector-transmitted diseases that are endemic in 88 tropical and subtropical countries (http://www.who.int/en/). Many geographical regions are endemic for multiple *Leishmania* species. This is the case in South America, where leishmaniasis are caused by at least eight different species of *Leishmania*, each one with different virulence and pathogenesis determinants and many of them displaying common areas of transmission [1]. New World

* Corresponding author. Centro de Biología Molecular Severo Ochoa (CSIC-UAM), Departamento de Biología Molecular, Nicolás Cabrera 1. Campus de Cantoblanco, Universidad Autónoma de Madrid, 28049 Madrid, Spain. Tel.: +34 91 1964508; fax: +34 91 1964420.
*E-mail address:* msoto@cbm.uam.es (M. Soto).
[1] These authors contributed equally to this work.

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leishmaniasis can be grouped into two broad clinical categories: visceral leishmaniasis (VL) and American tegumentary leishmaniasis (ATL). The latter category includes a variety of forms that are commonly referred to their characteristic clinical and pathologic features: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), and diffuse cutaneous leishmaniasis (DCL). VL is the most severe form of the disease, and in the New World it is caused by *Leishmania chagasi* infection. *Leishmania amazonensis* is capable of producing VL, although infection with this parasite is also associated with CL, MCL and DCL [1,2]. In this context, to be effective as a vaccine against American leishmaniasis, its components should be shared by different parasite species in different vertebrate hosts and the protective efficacy of these vaccine candidates should be analyzed in models of experimental infection caused by different parasite species.

Experimental leishmaniasis animal models have been employed for testing several candidate antigens in vaccine trials, although they may not be entirely predictive of how effective these vaccine candidates will perform in humans. Using mice models of CL and VL, it has been demonstrated that the main challenge for the development of an effective *Leishmania* vaccine is to find a formulation able to induce a Th1-type long-lasting immunity. This is primed by cytokines like IFN-γ, IL-12 and GM-CSF, produced by specific T cells and/or antigens presenting cells, but also controlling the disease associated, IL-4 mediated, humoral responses (mainly in CL models) and IL-10 deactivating responses [3,4]. From these experimental vaccine studies in mouse models, it is clear that some parasite fractions [5–7] or specific single proteins [8] are able to induce protection against *Leishmania* infection.

The antigenicity of the LRP has been demonstrated in dogs naturally infected by *Leishmania infantum* or *L. chagasi* [9]. In a previous work, we demonstrated that a subcellular fraction composed of the *Leishmania major* ribosomal proteins (LRP) administered in combination with a Th1 adjuvant (CpG oligodeoxynucleotides; CpG-ODN) was able to induce protection against *L. major* infection in C57BL/6 resistant and BALB/c susceptible mice [5]. In both mice strains, protection was correlated to an LRP-specific production of IFN-γ [5]. In BALB/c mice, protection was also correlated with a reduction of IL-4 and IL-10-mediated responses [5]. In this work we have investigated the role of an LRP based vaccine in the BALB/c experimental model using two different parasite species: *L. chagasi* and *L. amazonensis*.

*L. infantum* LRP extract was administered with saponin, an adjuvant capable of inducing Th1 immune responses and the production of cytotoxic T lymphocytes [10]. Saponin has been employed for the induction of immune responses in mice and dogs combined with *Leishmania* extracts [11,12] or recombinant proteins [13,14]. We show that the Th1 immune response induced by the co-inoculation of LRP plus saponin confers protection against the challenge with *L. chagasi* and *L. amazonensis* parasites in BALB/c mice. In both models, protection correlates to an LRP-specific and IL-12-dependent IFN-γ production mediated by $CD4^+$ and $CD8^+$ T cells and a diminished production of parasite-specific IL-4 and IL-10. Thus, we present evidence that these evolutionary conserved proteins combined with Th1-type adjuvants may be relevant in composing an effective pan-*Leishmania* vaccine.

## 2. Materials and methods

### 2.1. Mice and parasites

The Animal Use Committee of the Federal University of Minas Gerais (CETEA) (Brazil) approved experimental protocols. Female BALB/c mice (4–6 weeks old) were purchased from Institute of Biological Sciences, ICB, Federal University of Minas Gerais, Belo Horizonte, Brazil. *L. chagasi* (MOM/BR/1970/BH46), *L. amazonensis* (IFLA/BR/1967/PH-8) and *L. infantum* (MCAN/ES/1996/BCN/150/MON-1) parasites were grown at 24 °C in Schneider's (Sigma, St. Louis, MO, USA) medium supplemented with 20% heat-inactivated fetal bovine serum (FBS) (Sigma), 20 mM L-glutamine, 200 U/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml gentamicin, pH 7.4.

### 2.2. Antigen preparation

Soluble *Leishmania* antigenic (SLA) extract was prepared from stationary-phase promastigotes of *L. chagasi* and *L. amazonensis* growing in liquid culture, as previously described [15]. LRP was prepared from logarithmic phase promastigotes of *L. infantum*, as previously described [5].

### 2.3. Immunization, challenge infection, cutaneous lesion development and parasite quantitation

Mice ($n = 16$ per group) were subcutaneously immunized in their left hind footpads with 12 μg of LRP associated with 25 μg of saponin (*Quillaja saponaria* bark saponin) (Sigma), saponin alone or received saline. Three doses were administered in 2-week intervals. Four weeks after the final inoculation, eight animals per group were sacrificed for the analysis of the immune response elicited by vaccination. At the same time the remaining animals were s.c. infected, into the right hind footpad, with $10^5$ stationary-phase promastigotes of *L. chagasi* ($n = 4$, per group) or with $10^6$ stationary-phase promastigotes of *L. amazonensis* ($n = 4$ per group).

In mice infected with *L. amazonensis*, the course of the disease was monitored at weekly intervals by measuring footpad thickness with a metric calliper and expressed as the increase in thickness of the infected footpad compared to the uninfected footpad. At week eight post-challenge all animals were sacrificed and their spleens were harvested for parasite quantification and immunological analysis. Additionally, livers (in mice infected with *L. chagasi*) and infected skin fragments (in mice infected with *L. amazonensis*) were collected for parasite quantification. Sera samples were also collected before and after challenge for immunological analysis.

The number of parasites in the different tissues was determined by a limiting-dilution assay [16].

### 2.4. Cytokine production

Splenocyte cultures and cytokine assays were performed before and after challenge as described previously [15]. Briefly, single cell preparations from spleen tissue were plated in duplicate in 24-well plates (Nunc, Nunclon®, Roskilde, Denmark) at $2 \times 10^6$ cells/ml. Cells were incubated in DMEM medium alone (background control) or separately stimulated with SLA (50 μg/ml) or LRP (12 μg/ml) at 37 °C in 5% $CO_2$ for 48 h. IFN-γ, IL-4, IL-10, IL-12 and GM-CSF supernatants were assessed by sandwich ELISA using monoclonal antibodies specific for mouse cytokines (capture and detection) provided in commercial kits (Pharmingen, San Diego, CA, USA), following manufacturer's instructions. When indicated, and in order to block IL-12, $CD4^+$ and $CD8^+$ mediated T cell cytokine release, spleen cells stimulated with LRP (12 μg/ml) were incubated in the presence of 5 μg/ml of monoclonal antibody (mAb) against either mouse IL-12 (C17.8), CD4 (GK 1.5) or mouse CD8 (53-6.7). Appropriate isotype-matched controls: rat IgG2a (R35-95) and rat IgG2b (95-1) were also employed in the assays. Antibodies (no azide/low endotoxin™) were purchased from BD (Pharmingen).

### 2.5. Nitrite determination

Release of nitrite was determined in unstimulated or SLA-stimulated splenocyte cultures established at week 8 post-challenge. Following incubation for 48 h, 100 μl of culture supernatant was mixed with an equal volume of Griess reagent (Sigma). After an incubation of 10 min at room temperature, nitrite concentration was calculated using a standard curve of known concentrations. Data are expressed as μM per $2 \times 10^6$ cells per 48 h.

### 2.6. Analysis of the humoral responses

LRP- and SLA-specific IgG, IgG1 and IgG2a antibodies were measured by ELISA, as described elsewhere [15]. Peroxidase-labeled antibodies specific to mouse IgG, and mouse IgG1 or IgG2a isotypes (Sigma) were diluted at 1:5000 and incubated for 1 h at 37 °C, then incubated with $H_2O_2$ and o-phenylenediamine. Optical densities were read at 492 nm in a spectrophotometer.

### 2.7. Statistical analysis

The statistical analysis was made using the GraphPad Prism software (version 4 for Windows). All data comparisons were tested for significance by means of the one-way analysis of variance (ANOVA) using the Bonferroni's post-test for multiple comparisons of groups. $P$ values <0.05 were considered statistically significant ($^*P < 0.05$, $^{**}P < 0.005$). Data are representative of two independent experiments with similar results.

## 3. Results

### 3.1. Immunogenicity of the LRP combined with saponin in BALB/c mice

The immunogenicity of the *Leishmania* ribosomal proteins was evaluated in BALB/c mice after administration of the LRP in the presence of saponin. Following *in vitro* stimulation with LRP, spleen cells from the LRP plus saponin vaccinated mice produced significantly higher levels of IFN-γ, IL-12 and GM-CSF than those secreted by spleen cells from control mice, namely, saline and saponin groups (Fig. 1A). No increase in

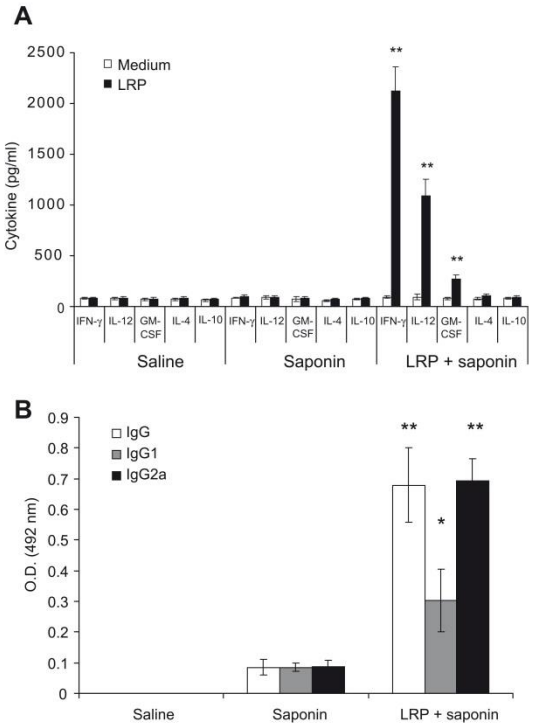


Fig. 1. Cytokine production and antibody response induced by vaccination in BALB/c mice. Spleen cells obtained from mice four weeks after the last vaccine dose were cultured *in vitro* and non-stimulated (medium; background control) or stimulated with LRP (12 μg/ml) for 48 h at 37 °C, 5% $CO_2$. IFN-γ, IL-12, GM-CSF, IL-4 and IL-10 levels were assessed by ELISA in culture supernatants. Each bar represents the mean ±SD of data from eight individual mice. Differences in the IFN-γ, IL-12 and GM-CSF levels between LRP plus saponin and the other two groups (saline and saponin) were statistically significant ($^{**}P < 0.005$) (A). Serum samples obtained from mice four weeks after the last vaccine dose were individually tested by ELISA to determine the presence of LRP-specific IgG, IgG1 and IgG2a antibodies. Each bar represents the mean ±standard deviation (SD) of data from four individual mice. Differences in the LRP-specific IgG ($^{**}P < 0.005$), IgG1 ($^*P < 0.05$) and IgG2a ($^{**}P < 0.005$) reactivity between the LRP plus saponin group and control (saline and saponin) groups were statistically significant (B). Data shown are representative of two independent experiments with similar results.

IL-4 and IL-10 production was observed after stimulation with LRP in any experimental groups. In addition, mice vaccinated with LRP plus saponin showed a specific anti-LRP humoral response that was predominantly of the IgG2a isotype (Fig. 1B).

### 3.2. Immunization with LRP plus saponin protects BALB/c mice against L. chagasi and L. amazonensis challenge

In order to evaluate the protective efficacy of LRP plus saponin vaccination against different forms of murine leishmaniasis, immunized mice were separately challenged by subcutaneous injection of two different *Leishmania* species. For the VL model, mice were infected with $10^5$ stationary-phase promastigotes of *L. chagasi*. Eight weeks after challenge, the parasite number in spleen and liver of infected mice was determined. Significant reduction of parasites was seen in mice immunized with LRP plus saponin as compared with those that received saline or adjuvant alone (Fig. 2A). The immunized mice showed a 6.7-log and 4.7-log reduction in the number of parasites in liver and a 12.3-log and 9.3-log reduction in spleen as compared with saline and adjuvant groups, respectively.

To determine the influence of the immunization with LRP plus saponin on *L. chagasi* specific killing effector function in the spleen of infected mice, nitrite was assayed as an indicator of nitric oxide (NO) production in macrophages. The nitrite production in splenocyte supernatants was significantly higher in mice vaccinated with LRP plus saponin after stimulation with *L. chagasi* SLA when compared with control groups that produced minimum amounts of this product (Fig. 2B).

For a model of CL, mice were infected by a high inoculum ($10^6$ stationary-phase promastigotes) of *L. amazonensis*. The development of dermal lesion was evaluated by measuring footpad swelling (Fig. 3A) and parasite loads (Fig. 3B) in the infected footpads and spleens of the mice. Animals immunized with LRP plus saponin displayed significant reductions in the footpad swelling, as compared to saline and saponin groups (30% and 60%, respectively) (Fig. 3A). In addition, immunized mice showed a 4.5-log and 3.2-log reduction in the number of parasites in spleen as compared with saline and adjuvant groups, respectively (Fig. 3B). Correlating to the lower parasite burden, nitrite production in splenocyte supernatants was significantly higher in mice vaccinated with LRP plus saponin than saline or saponin groups, after stimulation with *L. amazonensis* SLA (Fig. 3C).

### 3.3. Characterization of the cellular response elicited after infection

To determine the immunological parameters associated with protection induced in the mice by LRP plus saponin, the production of different cytokines in the supernatants of spleen cell cultures, established from the different mice groups and stimulated with *L. chagasi* or *L. amazonensis* SLA and *L. infantum* LRP after challenge was determined.

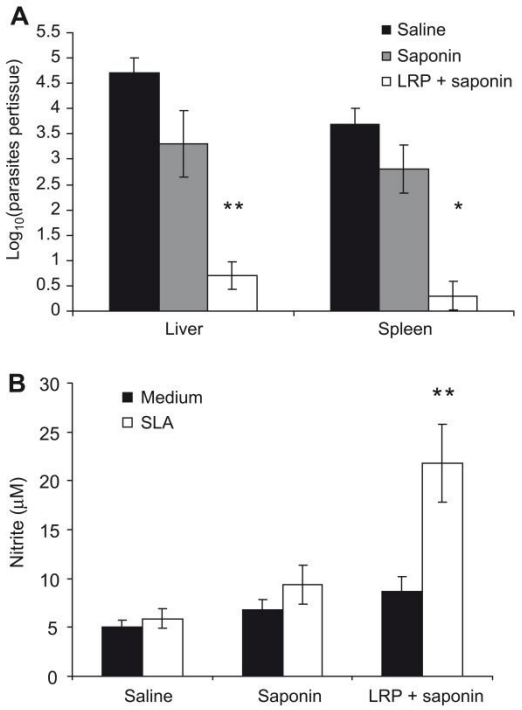


Fig. 2. Protection in BALB/c mice against *L. chagasi* infection by immunization with LRP plus saponin. Mice vaccinated with saponin or LRP plus saponin or inoculated with saline were s.c. challenged with $10^5$ promastigotes of *L. chagasi*. The number of parasites in the liver and spleen was measured eight weeks after infection. Mean ±standard deviation (SD) of four mice in each group is shown. Differences in the parasite load in the liver ($^{**}P < 0.005$) and spleen ($^{*}P < 0.05$) between LRP plus saponin and control (saline and adjuvant) groups were statistically significant (A). Determination of the amounts of nitrite in the supernatants of splenic cell cultures established from the three groups of mice, eight weeks after infection. Single cell suspensions were obtained from spleen of mice and were non-stimulated (medium; background control) or stimulated with SLA (50 μg/ml). Mean ±SD of nitrite levels determined in four individual mice per group is shown. Differences in nitrite levels between LRP plus saponin and saline and saponin groups were statistically significant ($^{**}P < 0.005$) (B). Data shown are representative of two independent experiments with similar results.

After *L. chagasi* infection, the spleen cells from mice vaccinated with LRP plus saponin produced higher levels of SLA- and LRP-specific IFN-γ than those secreted by spleen cells from both control mice groups at week eight after challenge (Fig. 4A). This data correlates well with the nitrite production pattern shown in Fig. 2B, since this killing effector molecule against leishmaniasis is produced by IFN-γ stimulated macrophages [17]. The contribution of $CD4^+$ and $CD8^+$ T cells and the dependence on IL-12 to the LRP- and SLA-specific production of IFN-γ in the spleen of the mice vaccinated with LRP plus saponin and infected were also analyzed. Production of IFN-γ was completely suppressed by anti-IL-12 or anti-CD4 monoclonal antibodies. The addition of anti-CD8

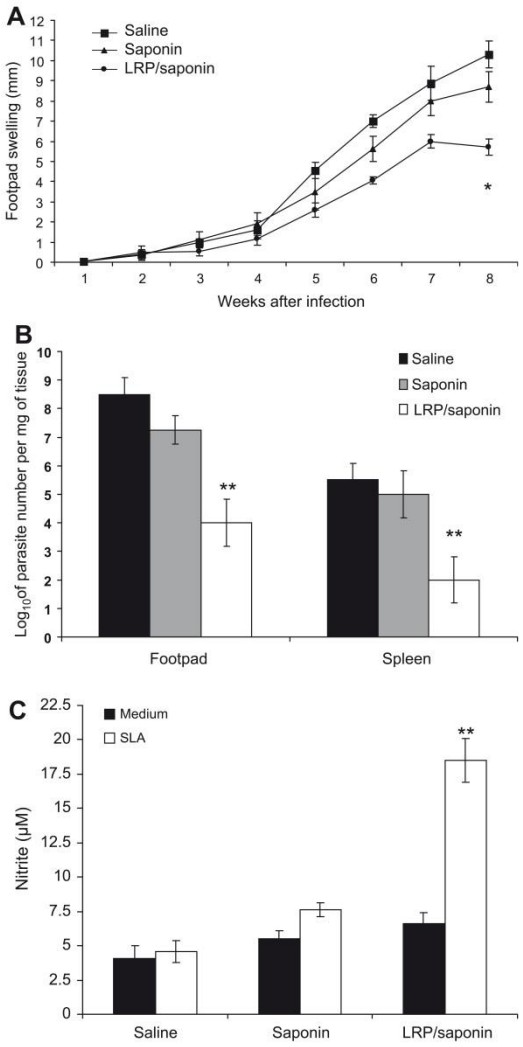


Fig. 3. Protection in BALB/c mice against *L. amazonensis* infection by immunization with LRP plus saponin. Mice vaccinated saponin or LRP plus saponin or inoculated with saline were s.c. challenged with $10^6$ stationary-phase promastigotes of *L. amazonensis*. Mean ±SD of four mice in each group is shown. Lesion development in the infected groups was monitored weekly until eight weeks after infection (A) Differences in the footpad swelling between LRP plus saponin and control (saline and adjuvant) groups were statistically significant at week eight after infection (*$P < 0.05$). Parasite burden determination in the infected footpads and spleens was analyzed eight weeks after infection (B). Differences in the parasite loads between LRP plus saponin and control (saline and adjuvant) groups were statistically significant (**$P < 0.005$). Determination of the amount of nitrite in the supernatants of splenic cell cultures established from the three groups of mice, eight weeks post-infection. Single cell suspensions were obtained from spleen of mice and were non-stimulated (medium: background control) or stimulated with SLA (50 μg/ml) (C). The mean ±SD of nitrite levels determined in four individual mice per group is shown. Differences in nitrite levels between LRP plus saponin and control groups were statistically significant (**$P < 0.005$). Data shown are representative of two independent experiments with similar results.

antibodies to the spleen cell cultures also induced a significant reduction in the amount of this cytokine in the supernatants, although levels were significantly higher than those observed in anti-IL-12 or anti-CD4 treated cultures (Fig. 4A). The observation that anti-CD4 and anti-CD8 inhibited production of IFN-γ is providing an indirect assessment of $CD8^+$ and $CD4^+$ T cell production of this cytokine, respectively. The ability of anti-CD4 antibody to completely inhibit production of IFN-γ should be indicating that $CD4^+$ T cells were providing a helper function for class I-dependent secretion of IFN-γ as it was previously reported in [18]. Altogether, our data may be indicating that co-inoculation of saponin and the LRP antigen induced both MHC class I and class II-restricted responses.

The IL-12 and GM-CSF production following *in vitro* stimulation with LRP or SLA was also analyzed eight weeks after *L. chagasi* challenge. Spleen cells from mice immunized with LRP plus saponin produced significantly higher levels of these cytokines after stimulation with both antigen preparations relative to control groups (Fig. 4B and C for IL-12 and GM-CSF, respectively). Finally, the SLA- or LRP-driven production of IL-4 (Fig. 4C) and IL-10 (Fig. 4D) was also analyzed after *L. chagasi* challenge. The LRP extract did not induce the production of these cytokines by the spleen cells of the three mice groups. Moreover, the SLA-specific production of IL-4 and especially IL-10 cytokines observed in the control groups was absent in the LRP plus saponin vaccinated mice.

Similar cytokines production profiles were observed eight weeks after *L. amazonensis* challenge infection. LRP plus saponin vaccinated mice spleen cells produced higher levels of IFN-γ (Fig. 5A), IL-12 (Fig. 5B) and GM-CSF (Fig. 5C) cytokines after *in vitro* stimulation with *L. amazonensis* SLA and LRP compared to saline and saponin mice groups. As observed in *L. chagasi* infected mice, the SLA- and LRP-specific secretion of IFN-γ was inhibited by anti-IL-12 or anti-CD4 monoclonal antibodies whereas treatment with anti-CD8 antibodies only partially reduced the level of this cytokine in the culture supernatants (Fig. 5A). The *L. amazonensis* SLA-specific production of IL-4 (Fig. 5D) and IL-10 (Fig. 5E) detected in spleen cells from control mice (saline and saponin groups) were significantly reduced in LRP plus saponin vaccinated mice. Remarkably, the level of SLA-induced IL-4 was higher in control mice infected with *L. amazonensis* (Fig. 5D) than control mice infected with *L. chagasi* (Fig. 4D).

### *3.4. Characterization of the anti-SLA humoral response after infection*

In BALB/c mice, the IL-4-dependent production of high titers of antibodies is associated with disease progression due to *L. amazonensis* [19] infection, but it is not certified in the *L. chagasi* murine model [20]. We analyzed the humoral responses elicited against SLA after infection in the different mice groups to compare the global anti-*Leishmania* antigens humoral response induced by infection with both *Leishmania* species. Very low levels of anti-SLA antibodies were observed in the sera of all mice groups infected with *L. chagasi*

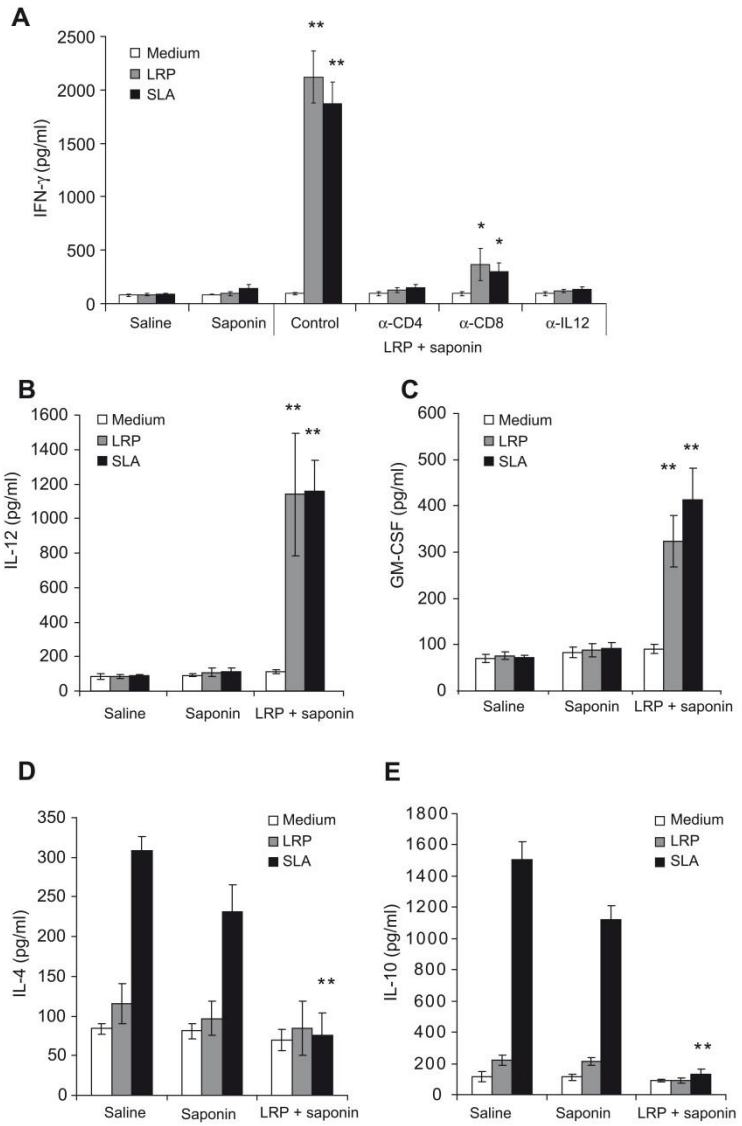


Fig. 4. Production of cytokines by spleen cells after *L. chagasi* challenge infection. Single cell suspensions were obtained from spleen of mice, eight weeks after infection. Cells were non-stimulated (medium; background control) or separately stimulated with *L. infantum* LRP (12 μg/ml) or *L. chagasi* SLA (50 μg/ml) for 48 h at 37 °C, 5% $CO_2$. IFN-γ (A) IL-12 (B), GM-CSF (C), IL-4 (D) and IL-10 (E) levels were measured in culture supernatants by ELISA. Mean ±SD of cytokine levels determined in four individual mice per group is shown. Differences in the levels of IFN-γ, IL-12 and GM-CSF between LRP plus saponin group and saline or saponin groups for SLA or LRP stimulus were statistically significant (**$P < 0.005$). Differences in the SLA-specific levels of IL-4 and IL-10 between LRP plus saponin group and saline or saponin groups were statistically significant (**$P < 0.005$). In panel A, the analysis of the involvement of IL-12 and T cells in the production of IFN-γ in mice vaccinated with LRP plus saponin is also shown. The level of IFN-γ was measured by ELISA in the supernatants of spleen cell cultures stimulated as above in the absence (control) or in the presence of either anti-IL-12, anti-CD4 or anti-CD8 monoclonal antibodies. When isotype-matched control antibodies were employed, the level of IFN-γ was similar to that observed in the absence of antibodies (data not shown). Differences between untreated cells and cultures treated with anti-CD4, anti-CD8 and anti-IL-12 monoclonal antibodies were statistically significant for both, SLA- and LRP-stimulated cells (**$P < 0.005$). Differences in the SLA- or LRP-specific IFN-γ production between anti-CD8 and anti-CD4 or anti-IL-12 treated cells were statistically significant (*$P < 0.05$). Data shown are representative of two independent experiments with similar results.

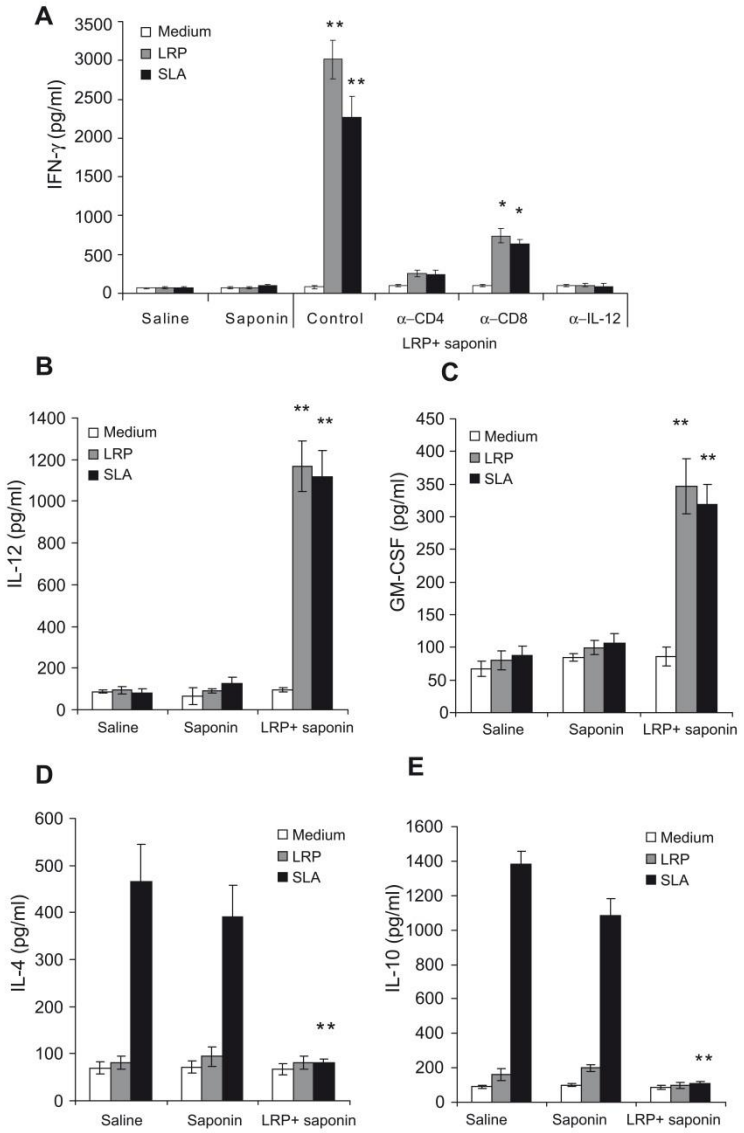


Fig. 5. Production of cytokines by spleen cells of BALB/c mice after *L. amazonensis* challenge infection. Single cell suspensions were obtained from spleen of mice, eight weeks after *L. amazonensis* infection. Cells were non-stimulated (Medium; background control) or separately stimulated with *L. infantum* LRP (12 μg/ml) or *L. chagasi* SLA (50 μg/ml) for 48 h at 37 °C, 5% $CO_2$. IFN-γ (A) IL-12 (B), GM-CSF (C), IL-4 (D) and IL-10 (E) levels were measured in culture supernatants by ELISA. Mean ±SD of cytokine levels determined in four individual mice per group is shown. Differences in the levels of IFN-γ, IL-12 and GM-CSF between LRP plus saponin group and saline or saponin groups for SLA or LRP stimulus were statistically significant (**$P < 0.005$). Differences in the SLA-specific levels of IL-4 and IL-10 between LRP plus saponin group and saline or saponin groups were statistically significant (**$P < 0.005$). In panel A, the analysis of the involvement of IL-12 and T cells in the production of IFN-γ in mice vaccinated with LRP plus saponin is also shown. The level of IFN-γ was measured by ELISA in the supernatants of spleen cell cultures stimulated as above in the absence (control) or in the presence of either anti-IL-12, anti-CD4 or anti-CD8 monoclonal antibodies. When isotype-matched control antibodies were employed, the level of IFN-γ was similar to that observed in the absence of antibodies (data not shown). Differences between untreated cells and cultures treated with anti-CD4, anti-CD8 and anti-IL-12 monoclonal antibodies were statistically significant for both, SLA- and LRP-stimulated cells (**$P < 0.005$). Differences in the SLA- or LRP-specific IFN-γ production between anti-CD8 and anti-CD4 or anti-IL-12 treated cells were statistically significant (*$P < 0.05$). Data shown are representative of two independent experiments with similar results.

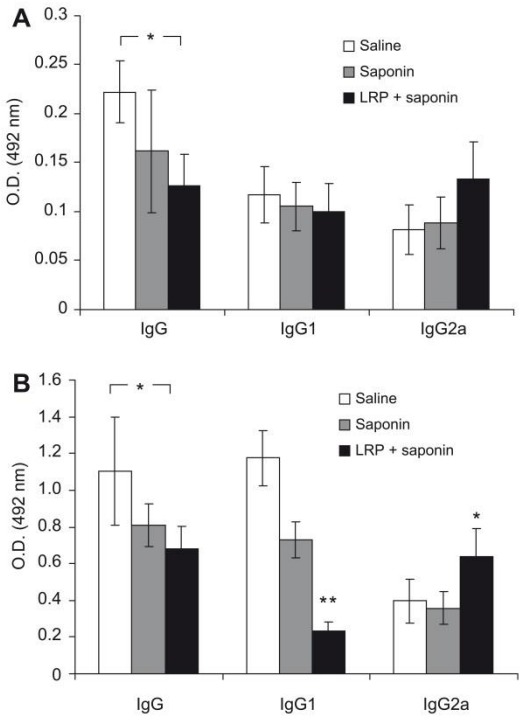


Fig. 6. Antibody response against SLA after *Leishmania* infection. Serum samples were obtained from mice infected with *L. chagasi* (A) or *L. amazonensis* (B), eight weeks after challenge. Sera were individually tested by ELISA to determine the presence of *L. chagasi* SLA-specific (A) or *L. amazonensis* SLA-specific (B) IgG, IgG1 and IgG2a antibodies. Each bar represents the mean ±SD of data from four individual mice per group. After *L. chagasi* infection, only differences in the SLA-specific IgG reactivity between saline and LRP plus saponin groups were found to be statistically significant (*$P < 0.05$). After *L. amazonensis* infection, differences in the SLA-specific IgG reactivity between saline and LRP plus saponin groups were found to be statistically significant (*$P < 0.05$). Also,IgG1 (**$P < 0.005$) and IgG2a (*$P < 0.05$) reactivity between protected mice and control (saline and saponin) groups were statistically significant. Data shown are representative of two independent experiments with similar results.

(Fig. 6A). We only found significant differences in the level of total IgG antibodies between saline and LRP plus saponin vaccinated mice, being lower in this last group. Similar levels of SLA-specific IgG1 and IgG2a antibodies were found in the three groups, the anti-SLA IgG2a antibodies being slightly higher in the LRP plus saponin vaccinated mice.

On the contrary, mice infected with *L. amazonensis* showed high levels of anti-SLA antibodies. Results shown in Fig. 6B indicate that immunization with LRP plus saponin conditioned the global *Leishmania*-specific humoral response induced by *L. amazonensis*, since antibodies produced by parasite-challenged mice vaccinated with LRP plus saponin were mainly of the IgG2a isotype, being the anti-SLA reactivity of the IgG1 isotype antibodies significantly lower than those detected in the other two groups.

## 4. Discussion

The development of vaccines against leishmaniasis requires the definition of potential candidates to be capable of inducing protective responses against different *Leishmania* species. In this way, many conserved intracellular *Leishmania* proteins like histones, ribosomal proteins, heat shock proteins, cysteine-proteinases and microtubule related proteins have been identified as antigenic and/or immunogenic in individuals suffering from different clinical forms of leishmaniasis [21,22]. Among them, *Leishmania* ribosomes can be considered as immunologically relevant particles during infection because some of their protein constituents are antigenic in human and dogs naturally infected with different *Leishmania* species [9,21,23]. In addition, some ribosomal proteins have been related to cell activities and cytokine release after infection in murine models resulting in dysfunction of the host immune system [21,24,25]. On the other hand, the administration of defined ribosomal antigens [26] or total parasite ribosomal proteins [5] using Th1 promoting adjuvants was able to induce protective responses in models of murine CL.

The purpose of this study was to analyze whether a preparation of *L. infantum* ribosomal proteins administered in combination with saponin was protective against *L. chagasi* and *L. amazonensis* challenge infection in BALB/c mice. We show that immunization with LRP extract obtained from *L. infantum* plus saponin was able to induce a predominant Th1 immune response against parasite ribosomal proteins. Vaccinated mice showed an *in vitro* LRP-specific production of IFN-γ and IL-12 combined with the presence of LRP-specific antibodies that were mainly of the IgG2a isotype, a humoral marker of Th1-type mediated responses [27]. In addition, very low levels of LRP-specific IL-4 or IL-10 were observed in the vaccinated mice.

After *L. chagasi* infection LRP plus saponin immunized mice displayed significant reduction of parasites in the spleen as well as in the liver when compared with mice vaccinated with the adjuvant alone or that received the saline solution employed in the vaccine formulation. The evaluation of parasite burden in both organs is an important marker of vaccine efficacy against VL, in view of the fact that an organ-specific immune response was observed after infection with viscerotropic *Leishmania* species following intravenous, intradermal or subcutaneous challenge in mice [20]. During the early stages of visceral infection in BALB/c mice, parasites multiply in large numbers in the liver, and afterwards the hepatic parasite load tends to decrease while parasitism in the spleen tends to increase. Thus, the liver can be considered an indicator of the initial multiplication of parasites and spleen as a reservoir for these microorganisms [28]. A reduction of parasite burdens was also observed in LRP plus saponin vaccinated mice compared with both control groups after *L. amazonensis* challenge. The decrease in the number of parasites found in the footpad was accompanied by a reduction of the dermal inflammatory lesions. Contrary to the lack of CL lesions found in BALB/c mice vaccinated with LRP plus CpG-ODN after *L. major* infection [5,29], mice vaccinated with

LRP plus saponin showed some footpad inflammation. Differences may be due to the different model of CL, since *L. amazonensis* parasites are more pathogenic than *L. major* [19]. Alternatively, the use of saponin instead CpG-ODN as adjuvant may be also implicated in the different degree of protection observed.

In both experimental models employed in this work, the decrease in number of parasites in the spleen from the LRP plus saponin vaccinated mice was correlated to the generation of NO, a molecule produced in activated macrophages that is critical to the control of parasite replication. The SLA-dependent production of NO observed in the spleen of the protected mice was correlated to an SLA-specific production of IFN-γ, a key cytokine implicated in the acquired immunity against infection with *Leishmania* species [28,30]. Our results show that IFN-γ was detectable in the supernatant of spleen cells established from the LRP plus saponin vaccinated mice after infection with both parasite species, and was rarely undetected in cultures established from control groups after *in vitro* re-stimulation. Both $CD4^+$ and $CD8^+$ T cells contribute to the production of IFN-γ in the protected mice. The vaccine-induced activation of both cell subsets may be important for the parasite killing observed in the spleen of protected mice, since the presence of both, antigen-specific Th1 responses and $CD8^+$ T cell responses, was related to protection against *Leishmania donovani* in mice vaccinated with a purified preparation of the promastigote surface proteins gp63 [31] or dp72 [32]. This was also observed in mice immunized with hybrid cell vaccines composed of macrophages expressing the kinetoplastid membrane protein KMP-11 [33] and in mice vaccinated with the recombinant hydrophilic acylated surface protein B1 [34]. In addition, the generation of *Leishmania*-specific $CD4^+$ and $CD8^+$ T cell responses have been correlated with control of infection in asymptomatic subjects infected with *L. infantum* [35]. In the protected mice, the IFN-γ production was IL-12-dependent and a parasite dependent production of IL-12 by spleen cells was detected after stimulation with parasite proteins. This cytokine has a central role in determining initial and late resistance to *Leishmania* infection [30,36] and has been also related with the generation of protective immunity against *L. infantum* in BALB/c mice vaccinated with membrane [37] or secreted [7] parasite fractions and in hamsters protected against the development of VL by vaccination with recombinant KMP-11 [38]. The protective role of IL-12 in VL may explain its ability to enhance the macrophage leishmanicidal activity [39,40].

Altogether, our data indicate that immunization with LRP plus saponin primed BALB/c mice for an LRP-specific Th1 immune response that was maintained after *L. chagasi* and *L. amazonensis* challenge, protecting vaccinated mice against development of VL and CL. These data are in agreement with those reported by Iborra et al. [5], demonstrating that the parasite Th1-specific immune response elicited after immunization with LRP extracts combined with CpG-ODN was able to induce protection in BALB/c and C57BL/6 mice against *L. major* infection. In BALB/c mice vaccinated with LRP plus CpG-ODN, protection against the development of CL was also correlated with a decrease of parasite-specific IL-4 and IL-10-mediated responses [5]. The decrease of these cytokine responses has also been related to the induction of vaccine-mediated protection against *L. chagasi* infection in BALB/c mice immunized with an A2 based DNA vaccine [41] and against *L. amazonensis* infection in the same mice strain after immunization with the *L. donovani* recombinant A2 protein administered with IL-12 [15]. Our results show that mice vaccinated with LRP plus saponin showed a similar control of these responses after *L. chagasi* and *L. amazonensis* infection. Thus, a significant decrease was observed in the *in vitro* production of SLA-specific IL-4 by spleen cells of the vaccinated mice after infection with both parasite species, when compared with both control mice groups. In *L. amazonensis* infected mice, the high levels of SLA-specific IL-4 production observed in control groups was correlated to the generation of high levels of anti-*Leishmania* antibodies. Thus, mice that received saline or saponin showed higher anti-SLA IgG1 antibody levels in comparison to IgG2a levels, whereas in the case of mice immunized with LRP plus saponin, the parasite-specific antibodies were mainly of the IgG2a isotype. The presence of high parasite-specific Th2 mediated humoral responses observed in control animals may have also contributed to disease progression, since *Leishmania* infection was impaired in the absence of circulating antibodies or in mice lacking the Fc receptors' common-γ chain [42]. Furthermore, $H\text{-}2^q$ syngeneic high- and low-antibody responder mice (Biozzi mice) were shown to be susceptible and resistant, respectively, to *L. amazonensis* infection [43].

Our results also show that protection against CL and VL progression in BALB/c mice was also associated with a remarkable, significant decrease of the IL-10-mediated immune responses. Very low levels of parasite-specific IL-10 production were detected after stimulation of the spleen cells from vaccinated mice eight weeks post-infection with both parasite species. However, spleen cells from control mice groups showed a significantly higher production of this cytokine. Regarding VL model, control of the parasite-mediated IL-10 responses in the vaccinated mice may be critical for protection, since IL-10 is considered as the most critical factor for VL progression after infection with viscerotropic *Leishmania* species [17,20] and IL-10 deficient BALB/c mice [44] or mice treated with an anti-IL-10 receptor antibody [45] are resistant to *L. donovani* infection. In addition, IL-10 has been also associated with CL disease due to *L. amazonensis* infection, since IL-10 deficient BALB/c mice had lower number of parasites in lesions than wild-type mice [46].

Finally, our results show that the spleen cells from protected mice produced higher levels of parasite-specific GM-CSF than controls, a cytokine related with macrophage activation and resistance against different intracellular pathogens including *L. major* [47] and *L. donovani* [48] in murine models. In addition, it has been shown that the immunization of human volunteers with a crude *Leishmania* preparation using GM-CSF as adjuvant induced parasite-specific Th1 responses [49] and that administration of a therapeutic vaccine containing four *Leishmania* antigens combined with GM-CSF

was correlated with resolution of mucosal lesions presented in an antimonial-refractory mucocutaneous leishmaniasis patient [50].

Results presented herein together with our previously reported data [5] indicate that *Leishmania* ribosomal proteins combined with Th1 adjuvants, like saponin, an adjuvant used in veterinary procedures can be used for the development of effective vaccines against leishmaniasis caused by viscerotropic and cutaneous *Leishmania* species. Taken together, our data confirm that high IFN-γ and IL-12 and low IL-4 and IL-10 levels are required for protection of BALB/c mice against *L. chagasi*, *L. amazonensis* and *L. major*. The association of LRP extracts or their defined ribosomal constituents with Th1 adjuvant systems might achieve these immune responses for the development of a pan-*Leishmania* vaccine.

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**Resumen del artículo: "Evaluation of immune responses and analysis of the effect of vaccination of the *Leishmania major* recombinant ribosomal proteins L3 or L5 in two different murine models of cutaneous leishmaniasis". Vaccine (V), 2013. 31:1312-1319.**

La posibilidad de caracterizar dentro del ribosoma aquellas proteínas que se asocian con las respuestas protectoras permitiría desarrollar vacunas de segunda generación más definidas que las basadas en purificaciones bioquímicas de los ribosomas. Con esta hipótesis, este trabajo describe la caracterización de cuatro de estas proteínas, su obtención como proteínas recombinantes y el estudio de su antigenicidad e inmunogenicidad.

Empleando los datos generados por el Proyecto de Secuenciación del Genoma de los Kinetoplástidos se identificaron los genes que de forma hipotética codificaban las siguientes proteínas ribosómicas en el genoma de *L. major*: LmS4, LmS6, LmL3 y LmL5. El clonaje de las correspondientes regiones codificantes en vectores de expresión procariota permitió obtener versiones recombinantes de estas proteínas. Se pudo confirmar su naturaleza ribosómica (mediante ensayos de "Western blot" con anticuerpos obtenidos gracias a las versiones recombinantes y extractos de LRP como fuente proteica), así como su naturaleza antigénica (al ser reconocidas por el suero de pacientes con LV caninas y humanas (Figura 1; V). Entre estas cuatro proteínas, LmL3 y LmL5 demostraron inducir el mayor grado de protección frente a la infección por *L. major* en ratones BALB/c si eran administradas junto a adyuvantes inductores de respuestas celulares (CpG-ODN) (Figura 2; V). Por ese motivo, el trabajo se focalizó en analizar las respuestas humorales y celulares generadas hacia estas dos proteínas tras la vacunación (Figura 3; V) y tras la infección (Figura 4; V). En resumen, la protección observada tras la inmunización con la proteína LmL5 se asoció con la generación de respuestas Th1, mientras que en los animales protegidos por la vacunación con la proteína LmL3 se observó un control de las respuestas mediadas por IL-10. Las respuestas humorales y celulares observadas frente a las proteínas totales del parásito en los animales vacunados y sus correspondientes controles tras la infección apoyan las observaciones anteriores (Figura 5; V). Se comprobó, además, que la inmunización de las proteínas combinadas con el adyuvante fue capaz de

inducir protección en un nuevo modelo experimental (ratones BALB/c infectados por *L. braziliensis* en presencia de la saliva de los vectores invertebrados) en el que otras formulaciones vacunales no generaron protección (Figura 6; V).

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# Evaluation of immune responses and analysis of the effect of vaccination of the *Leishmania major* recombinant ribosomal proteins L3 or L5 in two different murine models of cutaneous leishmaniasis

Laura Ramírez[a,1], Diego M. Santos[b,1], Ana P. Souza[b], Eduardo A.F. Coelho[c], Aldina Barral[b], Carlos Alonso[a], Marta R. Escutia[d], Pedro Bonay[a], Camila I. de Oliveira[b,1], Manuel Soto[a,*,1]

[a] Centro de Biología Molecular Severo Ochoa (CSIC-UAM), Departamento de Biología Molecular, Nicolás Cabrera 1, Universidad Autónoma de Madrid, 28049 Madrid, Spain
[b] Centro de Pesquisas Gonçalo Moniz (Fundação Oswaldo Cruz- FIOCRUZ) 40.296-710 Salvador, Bahia, Brazil
[c] Programa de Pós-Graduação em Ciências Saúde: Infectologia e Medicina Tropical, Faculdade de Medicina and Departamento de Patologia Clínica, COLTEC, Universidade Federal de Minas Gerais, 31.270-901, Belo Horizonte, Minas Gerais, Brazil
[d] Research & Development Department, Laboratorios LETI S.L.u., 28760 Madrid, Spain

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ABSTRACT

Four new antigenic proteins located in *Leishmania* ribosomes have been characterized: S4, S6, L3 and L5. Recombinant versions of the four ribosomal proteins from *Leishmania major* were recognized by sera from human and canine patients suffering different clinical forms of leishmaniasis. The prophylactic properties of these proteins were first studied in the experimental model of cutaneous leishmaniasis caused by *L. major* inoculation into BALB/c mice. The administration of two of them, LmL3 or LmL5 combined with CpG-oligodeoxynucleotides (CpG-ODN) was able to protect BALB/c mice against *L. major* infection. Vaccinated mice showed smaller lesions and parasite burden compared to mice inoculated with vaccine diluent or vaccine adjuvant. Protection was correlated with an antigen-specific increased production of IFN-γ paralleled by a decrease of the antigen-specific IL-10 mediated response in protected mice relative to non-protected controls. Further, it was demonstrated that BALB/c mice vaccinated with recombinant LmL3 or LmL5 plus CpG-ODN were also protected against the development of cutaneous lesions following inoculation of *L. braziliensis*. Together, data presented here indicate that LmL3 or LmL5 ribosomal proteins combined with Th1 inducing adjuvants, may be relevant components of a vaccine against cutaneous leishmaniasis caused by distinct species.



## 1. Introduction

Infection with *Leishmania* protozoan parasites can result in the development of leishmaniasis. Several species cause cutaneous leishmaniasis (CL) including *Leishmania major* and *L. braziliensis* (in the Old or New World, respectively); the latter is also related with development of mucocutaneous leishmaniasis (MCL). Visceral leishmaniasis (VL) is caused by the infection of *L. chagasi* in the New World or *L. infantum* and *L. donovani* in the Old World [1]. In natural and experimental leishmaniasis, effective primary immunity requires the production of IFN-γ by $CD4^+$ T cells and to a minor extend by $CD8^+$ T cells, which mediates nitric-oxide-depending parasite killing by the activation of infected macrophages [2]. Disease progression is related with the induction of humoral and IL-10 mediated responses [3].

A preparation of biochemically purified *Leishmania* ribosomal proteins (LRP) administered with CpG-oligodeoxynucleotides (CpG-ODN) conferred protection against challenge with *L. major* parasites in susceptible BALB/c and resistant C57BL/6 mice. Protection was correlated with a LRP-specific IL-12 dependent production of IFN-γ (in both mouse strains) and a diminished production of both IL-4 and IL-10 in BALB/c mice [4]. Also, BALB/c mice vaccinated with LRP plus CpG-ODN and subsequently infected were able to resist a secondary challenge [5]. A protective response against *L. chagasi* and *L. amazonensis* was observed in BALB/c mice when a LRP-specific IFN-γ mediated response was induced by administration of LRP combined with saponin [6].

In this work, four *L. major* ribosomal antigens have been characterized: LmS4, LmS6, LmL3 and LmL5. Their prophylactic properties were assayed in the *L. major*-BALB/c mouse model of CL. Since immunization of LmL3 or LmL5 combined with CpG-ODN elicited

*Abbreviations:* CpG-ODN, CpG-oligodeoxynucleotides; VL, visceral leishmaniasis; MCL, mucocutaneous leishmaniasis; CL, cutaneous leishmaniasis; LRP, *Leishmania* ribosomal proteins; SLA, soluble *Leishmania* antigens; MRP, mouse ribosomal proteins; CR, coding region; DLN, draining lymph nodes; OPD, ortophenylediaminedihydrochloride.

* Corresponding author. Tel.: +34 91 196 4471; fax: +34 91 196 4420.
*E-mail address:* msoto@cbm.uam.es (M. Soto).
[1] These authors contributed equally to this work.

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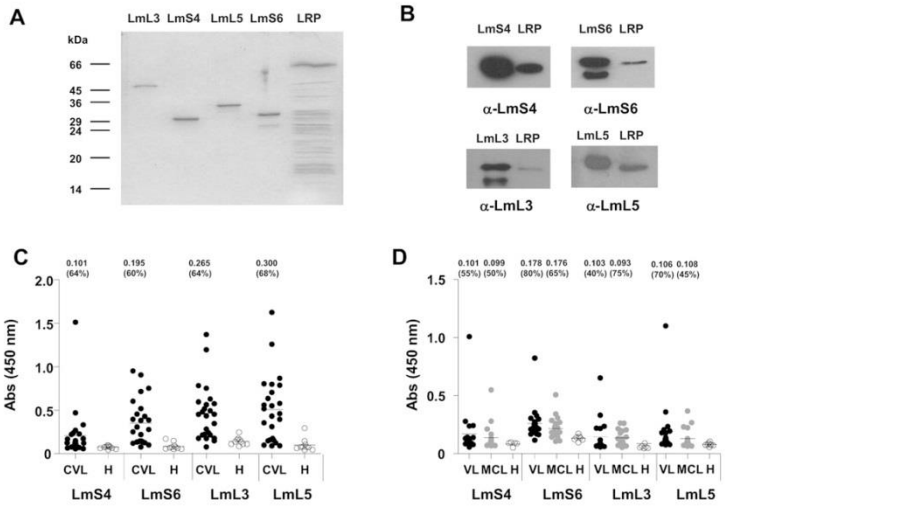



**Fig. 1.** Characterization of the LmS4, LmS6, LmL3 and LmL5 as antigenic components of the *Leishmania* ribosome. (A) One μg of each recombinant protein and 10 μg of *Leishmania major* LRP extracts were electrophoresed on a linear 10–13% gradient SDS-PAGE gel stained with Coomasie blue (B). Gels loaded with each one of the recombinant proteins and the LRP extracts were blotted and individually probed with the sera from mice immunized with LmS4, LmS6 and LmL3 (1/100) or with anti-LmL5 antibody fraction affinity purified from five canine VL sera. Horseradish peroxidase-conjugated anti-mouse (1/2000) or anti-dog IgG (1/2000) antibodies were used as the secondary reagents. (C) Antibody responses of canine VL (CVL, $n = 25$) and healthy animals' sera (H, $n = 8$) against the recombinant proteins. (D) Antibody response of human VL patients ($n = 20$), MCL ($n = 20$) and healthy individuals (H, $n = 8$) against the four recombinant proteins. All sera were tested for IgG reactivity by ELISA (1/200). Horseradish peroxidase-conjugated anti-dog (1/2000) or anti-human IgG (1/2000) antibodies were used as the secondary reagents. The OD value of each individual serum is shown. Bars represent means of each group. The cut-off value for negative and positive samples, calculated by comparison of the reactivity values from each group with the healthy sera using a Receiver-Operating Characteristic (ROC) analysis is indicated. It was defined as the lower O.D. value with a 100% of specificity. In brackets the percentage of positive sera is also indicated.

protective responses against *L. major* infection, their capacity to modulate *L. braziliensis* infection in the same mice strain was tested. Mice vaccinated with both proteins were able to control parasite growth in the site of infection in this New World species experimental model.

## 2. Materials and methods

### 2.1. Mice strains and parasites

Female BALB/c mice (6–8 week old) were purchased from Harlan (BCN, Spain) or were obtained from the Centro de Pesquisa Gonçalo Moniz, FIOCRUZ. Promastigotes of *L. major* strains (WHOM/IR/-/173) or clone V1 (MHOM/IL/80/Friedlin) and from *L. braziliensis* (MHOM/BR/01/BA788), were cultured at 26 °C in Schneider medium (Gibco, NY) supplemented with 10% fetal calf serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin.

### 2.2. CpG-ODN

Phosphorothioate-modified CpG-ODN (5′-TCAACGTTGA-3′ and 5′-GCTAGACGTTAGCGT-3′) were synthesized by Isogen (The Netherlands) and employed for their capacity to induce Th1 responses in mice when immunized with various leishmanial antigenic preparations [7,8].

### 2.3. Cloning of DNA sequences coding for L. major ribosomal proteins LmS4, LmS6, LmL3 and LmL5

The *L. major* LmS4, LmS6, LmL3 and LmL5 coding regions were obtained from the *L. major* genome database (www.genedb.org/genedb/leish) using the *Saccharomyces cerevisiae* orthologous protein sequences as probes [9]. Coding regions were PCR amplified using specific primers (Supplementary Fig. 1) and the DNA from *L. major* (MHOM/IL/80/Friedlin). Amplified DNAs were cloned into the pQE30 prokaryotic expression vector (Qiagen, Germany). The four clones were double-stranded sequenced in the same plasmid.

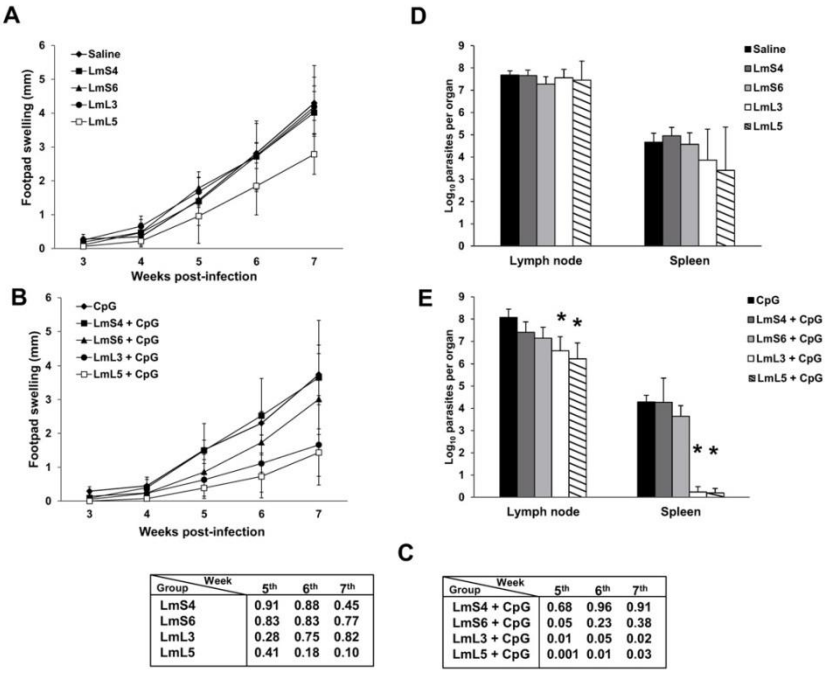
### 2.4. Protein purification

Recombinant proteins were over-expressed in *Escherichia coli*, purified under denaturing conditions onto Ni-nitrilotriacetic-acid-agarose columns (Qiagen) and refolded on the affinity column as described [10]. Polymyxin-agarose columns (Sigma, MO) were employed to remove residual endotoxin content (<10 pg of LPS per 1 μg of recombinant protein, measured by the Quantitative Chromogenic *Limulus* Amebocyte Assay QCL-1000 (BioWhittaker, MD)).

### 2.5. Sera, immunoblotting and ELISA assays

Mice sera were collected at the beginning of the experiment, before challenge with parasites and at the 7th week after challenge with *L. major*. Human VL and MCL sera were obtained from clinical and parasitologically diagnosed Brazilian patients. Canine symptomatic VL sera were collected in the Extremadura region of Spain [11]. Control sera were obtained from healthy individuals.

Soluble *Leishmania* antigens (SLA), mouse ribosomal proteins (MRP) and LRP were prepared as described in [4]. For immunoblotting, recombinant proteins and LRP extracts were electrophoresed and blotted as described in [11]. Anti-LmL3, anti-LmS4 or anti-LmS6 polyclonal sera were obtained from the immunized mice described below. Anti-LmL5 antibodies were obtained by passing canine VL sera through a recombinant LmL5 affinity chromatography column prepared as in [12]. For ELISA, recombinant proteins were used at 1.0 μg per well. Murine, canine and human sera were employed




| Group \ Week | 5th | 6th | 7th |
|---|---|---|---|
| LmS4 | 0.91 | 0.88 | 0.45 |
| LmS6 | 0.83 | 0.83 | 0.77 |
| LmL3 | 0.28 | 0.75 | 0.82 |
| LmL5 | 0.41 | 0.18 | 0.10 |

| Group \ Week | 5th | 6th | 7th |
|---|---|---|---|
| LmS4 + CpG | 0.68 | 0.96 | 0.91 |
| LmS6 + CpG | 0.05 | 0.23 | 0.38 |
| LmL3 + CpG | 0.01 | 0.05 | 0.02 |
| LmL5 + CpG | 0.001 | 0.01 | 0.03 |

**Fig. 2.** Course of *L. major* infection in BALB/c mice vaccinated with ribosomal proteins. Mice ($n = 6$ per group) were vaccinated with the indicated formulations and challenged in the footpad with $5 \times 10^4$ *L. major* promastigotes. Footpad swelling in groups vaccinated with the corresponding proteins without (A) or with (B) adjuvant is shown. Data correspond to the mean ± SD of the difference of thickness between the infected and the uninfected contra-lateral footpads. (C) *P* values (saline *versus* antigen or CpG-ODN *versus* antigen plus CpG-ODN) from data shown in panels A and B. The number of viable parasites (mean + SD) in the draining lymph node on the infected leg (popliteus) and in spleen were determined by limiting dilution at week seven post-challenge in mice vaccinated with the recombinant proteins without (D) or with (E) adjuvant. Serial dilution of each mouse sample was individually performed in triplicates. Comparison were established between each one of the vaccinated groups and their respective control (saline [D] and CpG-ODN [E]) (*$P < 0.05$ significant differences between vaccinated and control mice). Results in each panel are representative of ≥2 independent experiments.

as described in [4,12,13], respectively. Secondary antibodies were purchased from Nordic (Tilburg, The Netherlands). The reciprocal endpoint titre, defined as the inverse of the highest serum dilution factor giving an absorbance >0.15 was determined by serial dilution of the sera.

### 2.6. Immunizations, parasite challenge and parasite quantification

For the *L. major*-BALB/c model, mice ($n = 6$ per group) were independently inoculated in the right hind footpad with 10 μg of each recombinant protein (LmS4, LmS6, LmL3 or LmL5), alone or combined with 25 μg of each CpG-ODN. As control groups, mice were inoculated with 25 μg of each CpG-ODN or with saline. Each group was boosted two and four weeks later with the same dose. Parasite challenge was carried out by subcutaneous inoculation with $5 \times 10^4$ stationary-phase promastigotes of *L. major* (WHOM/IR/-/173) into the left footpad, four weeks after the last immunization. Footpad swelling was measured with a metric caliper (thickness of the left footpad minus thickness of the right footpad). For the *L. braziliensis* model BALB/c mice ($n = 5$ per group) were intradermically (i.d.) inoculated with 10 μg of each recombinant protein (LmL3 or LmL5) combined with 25 μg of each CpG-ODN or with a mixture of the proteins (5 μg each) plus 25 μg of each CpG-ODN in the left ear. Inoculation schedule was the same indicated above. Mice were challenged one month after the last inoculation in the dermis of the right ear with $1 \times 10^5$ stationary-phase promastigotes of *L. braziliensis* in the presence of sand fly saliva, as described [14]. Ear thickness was monitored weekly using a caliper. For parasite load determination the ears (in the case of *L. braziliensis* infection), draining lymph nodes (DLN) and spleen from each mouse were independently processed as described in Ref. [7] and assayed in triplicates by limiting dilution [15].

### 2.7. Measurement of cytokines

The release of IFN-γ, IL-10 and IL-4 was measured in culture supernatants of splenocytes or DLN cells obtained from the different mice groups, following stimulation with the corresponding recombinant proteins, using commercial ELISA kits (eBioscience, CA) as described [4]. Briefly, spleen or lymph node cells obtained from each mouse were seeded and independently cultured (at $5 \times 10^6$ cells per ml) during 48 h at 37 °C alone or with the next stimuli: recombinant LmL3 (10 μg/ml), recombinant LmL5 (10 μg/ml), SLA (12 μg/ml) or MRP (12 μg/ml). When indicated cells were stimulated with a mixture of the two recombinant proteins (5 μg/ml each one).

### 2.8. Statistical analysis

The Receiver Operating Characteristic (ROC) curves were used to analyze the data obtained with sera samples from patients. Statistical analysis with the vaccinated and infected mice was performed

by a two-tailed Student's *t*-test. Differences were considered significant when $P < 0.05$.

## 3. Results

### 3.1. Leishmania ribosomal proteins S4, S6, L3 and L5 are antigenic in canine and human leishmaniasis

The putative *L. major* S4, S6, L3 and L5 ribosomal proteins were identified using as probes the *S. cerevisiae* homologous aminoacid sequences [9] in a BLASTP search (Supplementary Fig. 2A). The degree of sequence identity with yeast and human ribosomal proteins (Supplementary Fig. 2A–B) supports the statement that they are components of ribosomes. In addition, antibodies specific for each protein revealed single bands with the expected molecular weights in a LRP preparation by Western blot (Fig. 1A and B). A high degree of sequence identity was observed for the proteins in different *Leishmania* species (Supplementary Fig. 2B).

The four recombinant proteins were recognized by the sera from dogs affected by VL (Fig. 1C). The percentages of positive sera ranged from 60 to 68% (Fig. 1C). They were also recognized by sera samples from Brazilian patients with VL and MCL, infected by *L. chagasi* and *L. braziliensis*, respectively (Fig. 1D).

### 3.2. Outcome of CL due to L. major following vaccination of BALB/c mice with recombinant ribosomal proteins

Next, we analyzed whether the immunization with the respective recombinant proteins was able to induce protection against *L. major* infection. Different groups of BALB/c mice were independently vaccinated with each recombinant protein in the absence or in the presence of CpG-ODN. Mice groups inoculated with the vaccine diluent (saline) or with the adjuvant alone were established as controls. After the challenge with *L. major* the course of infection was followed-up for 7 weeks (Fig. 2A in the absence and Fig. 2B in the presence of adjuvant). In the absence of adjuvant no significant differences in the footpad swelling between control and vaccinated groups were observed (Fig. 2C). When mice were immunized with the recombinant proteins combined with the adjuvant, the LmL3 plus CpG-ODN or LmL5 plus CpG-ODN groups showed a decrease in their lesion size compared to the control (CpG-ODN vaccinated mice) (Fig. 2B). Differences were significant from week 5 to week 7 (Fig. 2C). LmS6 plus CpG-ODN vaccinated mice showed a delay in the evolution of CL until week 5, but differences were not maintained at the end of the assay (Fig. 2B and C). Mice vaccinated with the proteins without adjuvant had a number of parasites similar to that of the saline control group mice in their DLN and their spleens (Fig. 2D). An approximately 2-log reduction in parasite burden was observed in the DLN cells from mice immunized with LmL3 plus CpG-ODN or LmL5 plus CpG-ODN, relative to the CpG-ODN control group (Fig. 2E). Moreover, spleens from mice immunized with LmL3 plus CpG-ODN or LmL5 plus CpG-ODN were almost free of parasites, contrary to the other vaccinated groups and controls (Fig. 2D and E).

The immune response induced by vaccination with LmL3 and LmL5 was analyzed before challenge. Co-administration of the antigens with the CpG-ODN adjuvant induced a Th1-biased immune response, which was absent in mice immunized with the proteins alone. A significantly higher antigen-specific production of IFN-γ was found after *in vitro* stimulation with the LmL3 ($P = 0.0000146$; $P = 0.00001468$) (Fig. 3A) and LmL5 ($P = 0.000015$; $P = 0.00029$) (Fig. 3B) proteins beside an IgG2a dominant antibody response against the vaccine antigen (Fig. 3C and D) when compared with saline and CpG-ODN mice group, respectively. Stimulation with SLA or MRP did not induce cytokine secretion by spleen cells (Fig. 3A and B).

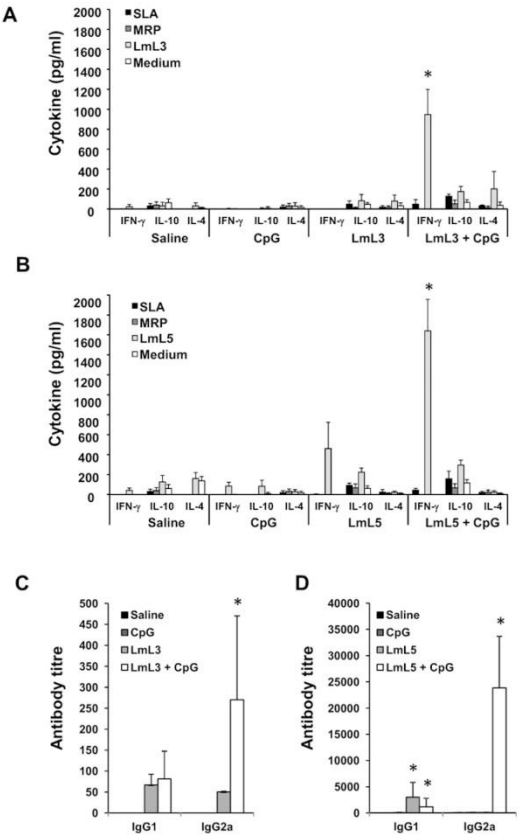



**Fig. 3.** Immune response elicited in mice vaccinated with LmL3 and LmL5. Mice ($n = 6$ per group) were vaccinated with LmL3 in the absence and presence of CpG-ODN (A) or LmL5 alone or plus CpG-ODN (B). Splenocytes from each mouse were independently cultured with medium alone or were *in vitro* stimulated with soluble *Leishmania* antigen (SLA), with mouse ribosomal proteins (MRP) and with the corresponding recombinant protein. Splenocytes from control mice, inoculated with saline or CpG-ODN, were stimulated with the same protein preparations in similar conditions. Culture supernatants were collected after 48 h and the levels of IFN-γ, IL-10 and IL-4 were independently measured by sandwich ELISA. Mean + SD are shown. (*$P < 0.05$ indicates a statistically difference between vaccinated mice relative to saline and CpG-ODN controls groups). Anti–LmL3 (C) and anti-LmL5 (D) IgG1 and IgG2a antibody titres were individually determined in the indicated mice groups ($n = 6$ per group) by ELISA. Sera were assayed from 1/100 to 1/200,000 and horseradish peroxidase-conjugated anti-mouse IgG1 (1/1000) or IgG2a (1/500) were used as the secondary antibodies. Mean plus SD are shown. (*$P < 0.05$ significant differences between vaccinated and control mice). Results in each panel are representative of ≥2 independent experiments.

Immune responses were also studied after challenge. Upon *in vitro* stimulation with the recombinant proteins, LmL3 plus CpG-ODN (Fig. 4A) or LmL5 plus CpG-ODN (Fig. 4B) vaccinated mice displayed a significant increase in the LmL3 or LmL5 driven IFN-γ production ($P = 0.00009$ and $P = 0.0005$, respectively) paralleled by a decrease in IL-10 secretion ($P = 0.009$ and $P = 0.00001$, respectively), relative to the CpG-ODN control group. These results are in accordance with the predominant IgG2a antigen-specific antibody response against LmL3 (Fig. 4C) and LmL5 (Fig. 4D), although

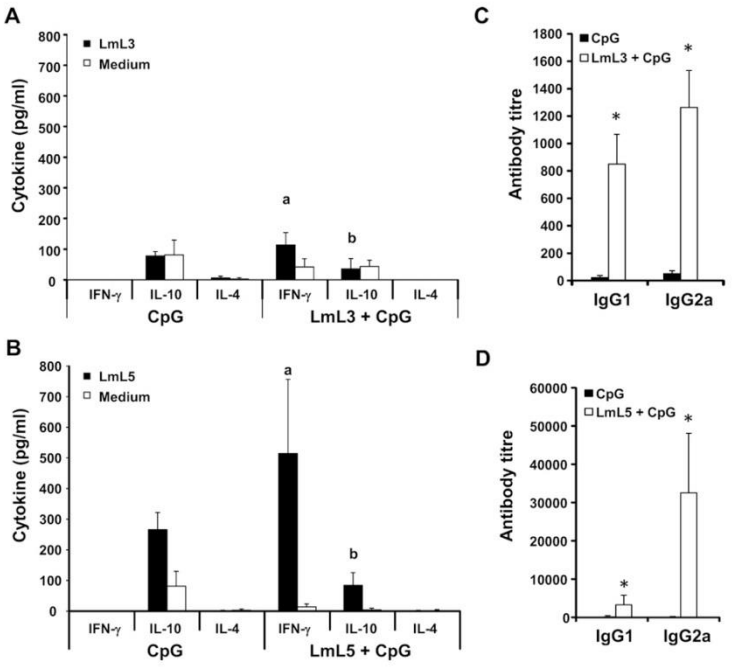



**Fig. 4.** Immune response elicited against LmL3 and LmL5 after *L. major* challenge in the protected mice. Mice ($n = 6$ per group) vaccinated with LmL3 plus CpG-ODN (A) or LmL5 plus CpG-ODN (B) were latter challenged with *L. major*. Splenocytes obtained from each mouse at week 7 after challenge, were independently cultured with medium alone or were *in vitro* stimulated with the corresponding recombinant protein. Control mice were inoculated with CpG-ODN and their splenocytes were stimulated as indicated. Culture supernatants were collected after 48 h and the level of IFN-γ, IL-10 and IL-4 was independently measured by sandwich ELISA. Mean + SD are shown. $P < 0.05$ indicates a statistically increase [a] or decrease [b] in the level of the indicated cytokine between vaccinated and CpG-ODN inoculated mice after infection. Anti-LmL3 (C) and anti-LmL5 (D) IgG1 and IgG2a antibody titres were individually determined in vaccinated and in CpG-ODN control mice ($n = 6$ per group) by ELISA. Sera were assayed from 1/100 to 1/200,000 and horseradish peroxidase-conjugated anti-mouse IgG1 (1/1000) or IgG2a (1/500) were used as the secondary antibodies. Mean plus SD are shown. (*$P < 0.05$ indicates significant differences between vaccinated and CpG-ODN control mice after infection). Results in each panel are representative of 3 independent experiments.

anti-LmL3 and anti-LmL5 IgG1antibodies were also detected (Fig. 4C and D).

The cellular response against SLA was also analyzed in the protected mice after challenge. Secretion of IFN-γ was significantly higher in LmL3 plus CpG-ODN or LmL5 plus CpG-ODN vaccinated mice, when compared to mice inoculated with CpG-ODN ($P = 0.014$ and $P = 0.017$, respectively) (Fig. 5A). In LmL3 plus CpG-ODN vaccinated mice a decrease in the SLA-dependent IL-10 production was also observed when compared with CpG-ODN immunized mice ($P = 0.009$). An increment in the SLA-specific IgG2a antibodies was

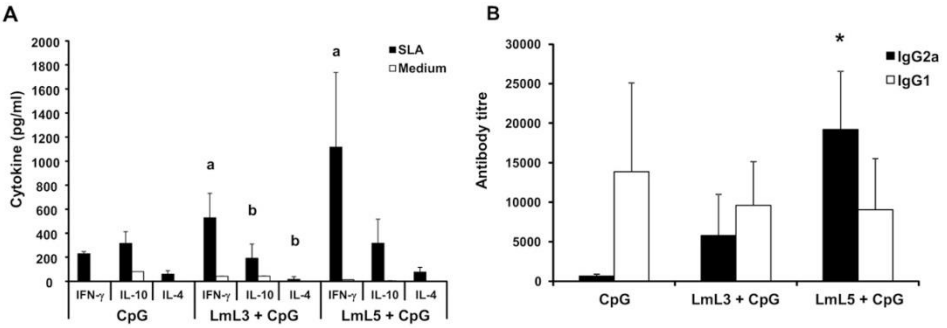



**Fig. 5.** Immune response elicited against soluble leishmanial antigens (SLA) after *L. major* challenge in the protected mice. (A) Mice ($n = 6$ per group) were vaccinated with LmL3 plus CpG-ODN or with LmL5 plus CpG-ODN. Mice were later challenged with *L. major* and splenocytes, obtained from each mouse at week 7 after challenge, were independently cultured with medium alone or were *in vitro* stimulated with SLA. Control mice were immunized with CpG-ODN and their splenocytes were cultured as indicated in the presence or in the absence of SLA. Culture supernatants were collected after 48 h and the levels of IFN-γ, IL-10 and IL-4 were independently measured by sandwich ELISA. Mean + SD are shown. ($P < 0.05$ indicates a statistically increase [a] or decrease [b] in the level of the indicated cytokine between vaccinated and CpG-ODN inoculated mice after infection). (B) Anti-SLA IgG1 and IgG2a antibody titres were individually determined in vaccinated and in CpG-ODN control mice ($n = 6$) by ELISA. Sera were assayed from 1/100 to 1/200,000 and horseradish peroxidase-conjugated anti-mouse IgG1 (1/1000) or IgG2a (1/500) were used as the secondary antibodies. Mean plus SD are shown. (*$P < 0.05$ indicates significant differences between vaccinated and CpG control mice after infection). Results in each panel are representative of 3 independent experiments.

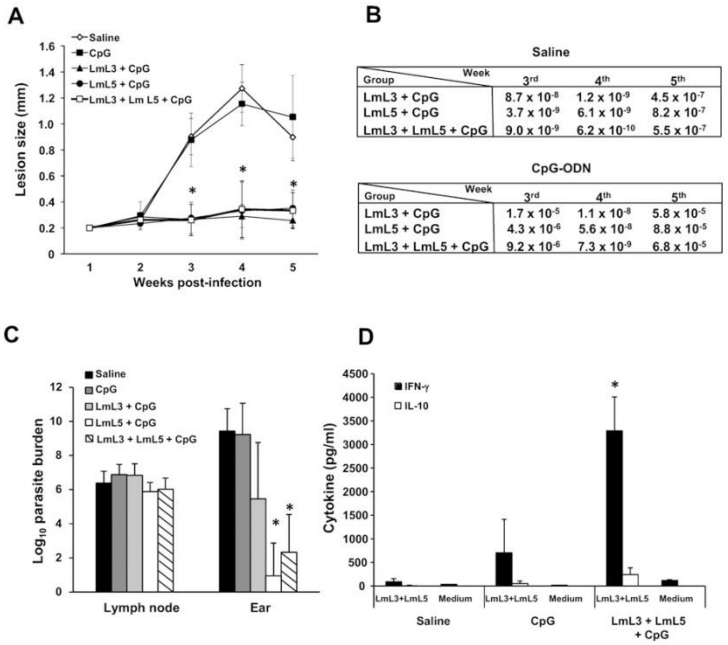



**Fig. 6.** Course of *L. braziliensis* infection in BALB/c mice vaccinated with LmL3 and LmL5. Mice were vaccinated with LmL3 plus CpG-ODN, with LmL5 plus CpG-ODN or with LmL3 plus LmL5 plus CpG-ODN and challenged in the ear with $1 \times 10^5$ *L. braziliensis* promastigotes plus salivary gland sonicate. (A) Course of CL development expressed as the mean ± SD of the ear thickness from five mice (*$P < 0.05$ indicates significant differences between vaccinated and saline or CpG-ODN controls). (B) *P* values (saline or CpG-ODN *versus* antigens plus CpG-ODN) from data shown in panel A. (C) Parasite burden in the ear dermis was independently quantified at 5th week five post-infection. Results are expressed as the mean + SD of five ears per group (*$P < 0.05$ indicates a significant decrease between mice vaccinated with LmL5 plus CpG-ODN or LmL3 plus LmL5 plus CpG-ODN and control mice inoculated with CpG-ODN or with saline). (D) Production of IFN-γ and IL-10 by lymph node cells following *in vitro* stimulation with a mixture of LmL3 and LmL5 (5 μg each protein) five weeks after challenge. Samples were independently processed and the level of cytokines are expressed as the mean plus SD of five mice per group (*$P < 0.05$ indicates a significant increase in IFN-γ levels between rLmL3 plus rLmL5 plus CpG-ODN group *versus* CpG-ODN or saline mice groups). Results in each panel are representative of 2 independent experiments.

observed in LmL3 plus CpG-ODN and LmL5 plus CpG-ODN groups relative to CpG-ODN group, only significant in LmL5 plus CpG-ODN group ($P = 0.014$) (Fig. 5B). The IgG1 response to SLA was similar between the three groups (Fig. 5B).

### 3.3. Vaccination with LmL3 plus CpG-ODN and LmL5 plus CpG-ODN protects BALB/c mice against L. braziliensis challenge

We also investigated whether immunization with LmL3 or LmL5 was able to confer protection in an experimental model of New World CL. Mice were independently vaccinated with LmL3 or LmL5 combined with CpG-ODN or with a mixed formulation of LmL3 and LmL5 plus CpG-ODN. The outcome of infection with *L. braziliensis* inoculated in the presence of insect vector saliva was evaluated. Ear lesions of vaccinated mice groups were significantly smaller to control groups (saline or CpG-ODN) (Fig. 6A and B). Parasite burden in the ear dermis of the three vaccinated groups was lower than the parasite load of the control groups. Differences with both control groups were significant in the LmL5 plus CpG-ODN ($P = 0.00016$) and in the LmL3 plus LmL5 plus CpG-ODN groups ($P = 0.005$) (Fig. 6C). Similar parasite burden was detected in the DLN of controls and vaccinated mice (Fig. 6C). Finally, to analyze the cellular response elicited against the vaccine antigens, DLN cells from mice immunized with the mixed formulation and both control groups were stimulated with a mixture of the LmL3 and LmL5 recombinant proteins. A significant increase in antigen-specific IFN-γ production was observed in the protected mice relative to both control groups ($P = 0.015$ for saline and $P = 0.007$ for CpG-ODN).

## 4. Discussion

Given that vaccines based on *Leishmania* ribosomal preparations have induced protection against disease development when immunized with Th1 inducing adjuvants [4–6] we have moved toward the identification of protective antigens in the *Leishmania* ribosome.

A few ribosome structural proteins have been described as antigenic in canine or human leishmaniasis, including the P0 [16,17], the L6 [18] and, recently, the L25 and L23a proteins [19]. The four ribosomal proteins identified herein (S4, S6, L3 and L5) are antigenic in different forms of the disease caused by distinct parasite species such as *L. chagasi* and *L. braziliensis* (in humans) and *L. infantum* (in dogs). Although the recombinant proteins were obtained from *L. major* DNA the high degree of sequence conservation existing between *Leishmania* parasite ribosomal orthologues may explain the observed cross-reactivity. Even though it was observed some variability in the recognition between human and canine VL sera (also between VL and MCL human patients) as occur with other parasite antigens [20], our data allow to conclude that the four studied proteins interact with the host immune system, in spite of differences in parasite species and disease forms.

Next, the protective capacities of the characterized antigens were evaluated. Previous reports have identified the prophylactic capacities of three structural proteins of the parasite ribosome: P0 [7], L22 and S19 [21]. Combination of the CpG-ODN adjuvant with the LmL3 or LmL5 proteins induced an immune state that was able to control CL disease due to *L. major* infection in susceptible

BALB/c mice. The immune correlate of protection was the induction of a Th1-like response specific for the recombinant LmL3 or LmL5 proteins. The magnitude of antigen dependent IFN-γ secretion and antigen specific IgG2a titers were higher in the LmL5 plus CpG-ODN vaccinated mice than in mice immunized with LmL3 plus CpG-ODN based vaccine. Antigen specific Th1 responses induced upon vaccination were maintained after infection. Vaccines also controlled the LmL3 and LmL5-driven IL-10 responses induced after infection. However, the presence of IgG1 antibodies specific for LmL3, LmL5 is indicating that the Th2 immune response elicited against them by *L. major* infection was not completely abrogated by the vaccines. The Th2-biased response induced after infection against LmL3 and by LmL5 proteins occurring in human or dogs was also observed in mice vaccinated with the proteins without the adjuvant, since in these mice antibodies elicited against both antigens were of the IgG1 isotype (data not shown). This fact points out the importance of the adjuvant in ribosome-based vaccines. Thus, CpG-ODN motifs were able to redirect toward a protective Th1-like profile the response against LmL3 and LmL5 as also occur with other vaccine formulations like these based on LmSTI1 plus CpG-ODN soluble and particulate preparations [22].

Although the protective effects of the LmL3 or the LmL5 plus CpG-ODN documented here were analyzed in a model that employ a syringe-based challenge the results obtained allowed us to conclude that the protective effect of the immunization of LmL3 and LmL5 based vaccines is similar to that obtained with various parasite proteins assayed for protection under similar conditions [23].

Cross-prophylactic properties of the LmL3 or LmL5 based vaccines were also tested in an experimental model of CL caused by *L. braziliensis* [24]. Many of the proteins known to induce protection against *L. major* or *L. infantum* infection in BALB/c mice, were not able to control the CL caused by *L. braziliensis* [25,26] or only induce partial protection [27]. In this model, BALB/c mice show lesions in the challenge site (ear) that are resolved after induction of a Th1 type immune response that eliminates parasites from ears, maintaining a chronic infection in the DLN [24]. Since co-inoculation of vector saliva and *L. braziliensis* led to a significant exacerbation of both lesion size and parasites load in the mice experimental model [28] we have employed here a syringe-based challenge in which vector saliva and stationary parasites are co-inoculated. Interestingly, we found that ear inflammatory lesions were almost absent in vaccinated mice and a very low number of parasites was detected in the ears 5 weeks after challenge, especially in mice vaccinated with LmL5 plus CpG-ODN or with a combination of both ribosomal proteins and CpG-ODN. Given the antigen specific IFN-γ mediated response was observed in protected mice, it can be suggested that IFN-γ-secreting cells may have migrated to the infected ear early after challenge, promoting parasite killing in the absence of an inflammatory response of a high magnitude. These cells, however, are unable to destroy parasites in the DLNs as also occur in the infected controls in accordance to what it has been previously reported for this experimental model of infection [24]. Since results obtained by our group have shown that immunization with LmL3 or LmL5 ribosomal antigens combined with CpG-ODN also reduced parasite loads in BALB/c mice infected with *L. chagasi* (manuscript in preparation), we conclude that the LmL3 and LmL5 antigens, formulated with Th1 inducing adjuvants should be considered in the development of vaccines against leishmaniasis.

## 5. Conclusions

In this work four new antigenic proteins have been described in *Leishmania* ribosome: S4, S6, L3 and L5. Recombinant proteins obtained from *L. major* were recognized by the sera from individuals infected with different parasite species and suffering different forms of the disease. Two of them, LmL3 or LmL5 were able to protect mice against CL caused by *L. major* and by *L. braziliensis* when administered in the presence of a Th1 inducing adjuvant. In both models, protection was associated with the induction of antigen-specific IFN-γ mediated responses, but also with control of the antigen dependent production of IL-10 in some cases. Altogether, data presented here are indicating that LmL3 and LmL5 may be considered relevant antigens in the formulation of vaccines against leishmaniasis.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2012.12.071.

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**Supplementary Figure 1.** Description of the primers employed for PCR amplification.

**LmS4** (acc. number LmjF13.1220).

Sense 5'- CGGGATCCATGGCCAAGAAGCACCTCAAG-3' (*Bam*HI).

Antisense 5'-CCCAAGCTTTCACTTGCGGGCCCTGCGGG-3' (*Hin*dIII).

**LmS6** (acc. number LmJF35.2010).

Sense 5'-CGGGATCCATGAAGCTCAACATCGCGTAC-3' (*Bam*HI).

Antisense 5'-CCCAAGCTTACTTCTTCTGGAATGCTG-3' (*Hind*III).

**LmL3** (acc. number LmjF34.2880).

Sense 5'-CGGGATCCATGTCTCACTGCAAGTTCGAG-3' (*Bam*HI).

Antisense 5'-AACTGCAGTTACTTCTTCGCGGCCTTTG-3' (*Pst*I).

**LmL5** (acc. number LmjF35.1890).

Sense 5'-CGGGATCCATGTGCACGCTGGCAAATTG-3' (*Bam*H1)

Antisense 5'-CCCAAGCTTTTACTTGCCGAGGCGCTCGC-3' (*Hind*III).

**Supplementary Figure 2.**

**A) *Leishmania major* aminoacid sequences of the proteins included in this work.**

**LmS4**

```
MAKKHLKRLYAPKDWMLSKLTGVFAPRPRPGPHKLRECLPLLVIIRNRLKYALNAREGEM  60
ILRQGLVHVDNHPRRDGKYPAGFMDVVEIPKTGDRFRLMYDVKGRFALVNLSEAEAQIKL 120
MKVVNLYTATGRVPVAVTHDGHRIRYPDPHTSIGDTIVYNVKEKKCVDLIKNRQGKAVIV 180
TGGANRGRIGEIVKVECHPGAFNIAHLKDASGAEFATRAANIFVIGKDLNNLQVTVPKQQ 240
GLRMNVIQEREERLIAAEARKNAPARGARRARK                              273
```

Identity: 100% to the protein with acc. number LmjF13.1220.

49% to the *Saccharomyces cerevisiae* S4 protein (YJR145c)

Mw: 30.68 kDa

pI: 10,98

**LmS6**

```
MKLNIAYPRNGTVKQFEISDEVLRRVQLQDYRLGNEVDGAIFGSEFKGYIFRLRGGSDKD  60
GFPMVPGVLASSRVSLLVKRGAIGFNTFRGYQGERRRKNVRGCVLASDIALVNVTISKVG 120
DQPIEGVTDTTAPRRLGPKRASKIRKLFNLSRTEDVRKYVVRRRVVKSGKKDRLKAPKIQ 180
RLITPRVKARRAKKAKDAIAKVRASAAERREYLRLIASNRRALRQRDHSKKHTRKVHAQR 240
AEVAAFQKK                                                      249
```

Identity: 100% to the protein with acc. number LmjF35.2010.

52% to the *S. cerevisiae* S6 protein (YPL090c)

Mw: 28.30 kDa

pI: 11.98

**LmL3**

```
MSHCKFEHPRHGHLGFLPRKRSRQIRGRARAFPKDDATQKPHLTSFMVFKAGMTHIVRDV  60
DRPGSKVNKKEVVEPVTILEAPPMVIVGIVGYRQTPVGLKTIGTVWAHHTSVEFRRRYYK 120
NWKQSAQLAFSRQKQFANTKEGKVAEARTLNAFAKKASVIRVIAHTQLRKLRNHRVGVKK 180
AHVQEIQVNGGSVAAKIALAKSLLEKEVRVDSVFQQSEACDVCSVTKGHGTEGVVKRWGV 240
ACLPRKTHRGLRKVACIGAWHPARVMYTVARAGQHGYHHRTQLNKKIYQIGRSVAVEPNQ 300
ATTTYDLTAKTITPMGGFVGYGTVRNDYVMLKGSVSGPRRRVMTLRRPMAPQTSRQLKEK 360
IVLKFIDTSSKIGHGRFQTKKEKNQWFGPLKKDRIRREERLRKERAARAVERKAKAAKK  419
```

Identity: 100% to the protein with acc. number LmjF34.2880.

53% to the *S. cerevisiae* L3 protein (YOR063w)

Mw: 48.9 kDa

pI: 11.69

**LmL5**

```
MCTLANWVRAIIKKHSTLAHTLEMPFVKVVKNKAYFKRFQVKYRRRREGKTDYHARRQMV  60
LQDKTKFGSPKYRLVVRITNKDIIAQIVQAKIVGDEVVMAAYAHELPAFGIEHGLTNYAA 120
AYATGLLLARRTLAKLGIADKFQGAKEADGSYSAVRTKKDDEGDDEERFPFKAILDVGLA 180
RTTTGARVFGVLKGAVDGGMAVPHRPNRFPGYNKEKSSLDAKVHRDRIFGKHVADYLKQV 240
KEEASSNPDEKCVQFSKYMAAKVLPESIEGMYKKAHAAIRADPSKSLPKKAKKEGVAHKS 300
YKTKKLSGAEKRAAAKAKVAAIRERLGK                                  328
```

Identity: 100% to the protein with acc. number LmjF35.1890.

50% to the *S. cerevisiae* L5 protein (YPL131w)

Mw: 36.6 kDa

pI: 10.29

**B) Homology degree analysis.**

| *L. major* | *L. braziliensis* | *L. infantum* | *Homo sapiens* |
|---|---|---|---|
| **S4** | 263/273 (96.3%) | 270/273 (98.9%) | 136/257 (52.9%) |
| | 269/273 (98.5%) | 273/273 (100%) | 177/257 (68.9%) |
| **S6** | 239/249 (96.0%) | 248/249 (99.6%) | 115/249 (46.2%) |
| | 246/249 (98.8%) | 249/249 (100%) | 160/249 (64.3%) |
| **L3** | 179/206 (86.9%)* | 418/420 (99.5%) | 240/404 (59.4%) |
| | 189/206 (98.8%)* | 418/420 (99.5%) | 299/404 (74.1%) |
| **L5** | 289/305 (94.8%) | 303/305 (99.3%) | 161/305 (52.8%) |
| | 297/305 (97.4%) | 305/305 (100%) | 210/305 (68.9%) |

Identity (top) and similarity (below) between protein orthologous proteins are indicated in each cell. Sequences employed in this table were obtained from the corresponding sequence database, and have the next accession numbers: *L. major*: S4 (LmjF13.1220); S6 (LmjF35.2010); L3 (LmjF34.2880); L5 (LmjF35.1890). *L. braziliensis*: S4 (LbrM13_V2.1000); S6 (LbrM33_V2.0840); L3 (LbrM20_V2.2480)*: L5 (LbrM34_V2.1790). *L. infantum*: S4 (LinJ13_V3.1129); S6 (LinJ21_V3.2510); L3 (LinJ32_V3.3320); L5 (LinJ35_V3.1870). *Homo sapiens*: S4 (NP_033120.1); S6 (NP_033122.1); L3 (NP_038790.2); L5 (NP_058676.1). *For *L. braziliensis* only L3 N-terminal regions are available in the database. A high degree of homology exist between *Mus musculus* and *Homo sapiens* ribosomal proteins (identities ranging between 98.3 to 100% for the proteins included in this work).

**Resumen del artículo: "Cross-protective effect of a combined L5 plus L3 *Leishmania major* ribosomal protein based vaccine combined with a Th1 adjuvant in murine cutaneous and visceral leishmaniasis". Parasites & Vectors (P&V), 2014. 7:3.**

Tras la identificación de dos proteínas ribosómicas (LmL3 y LmL5) capaces de generar protección frente a la leishmaniosis cutánea causada por *L. major* y *L. braziliensis*, se planteó ampliar el estudio del potencial protector con otros modelos adicionales de infección en el ratón. Con esta motivación, se realizaron ensayos de protección de una formulación combinada de ambos antígenos y el adyuvante CpG-ODN empleando ratones BALB/c y las dos especies del parásito ya empleadas en los ensayos de vacunas basadas en LRP: *L. chagasi* y *L. amazonensis*.

En este trabajo se analizó en primer lugar la respuesta inmune generada por la vacuna de combinación y posteriormente el efecto de la vacunación en la progresión de la LV provocada por la infección de *L. chagasi* y la LC causada por *L. amazonensis* La producción de IFN-γ tras la estimulación de las células del bazo de los animales vacunados con los antígenos de la vacuna y la presencia de anticuerpos de la subclase IgG2a específicos de ambas proteínas demostraron la generación de una respuesta del tipo Th1 (Figura 1; P&V). Es interesante destacar que la intensidad de la respuesta fue diferente, resultando mucho más inmunogénica la proteína LmL5 que la proteína LmL3. Esta respuesta se asoció con una moderada reducción de los síntomas clínicos (LC causada por *L. amazonensis*) y la disminución de carga parasitaria en ambas infecciones (Figura 2: P&V). El análisis de las respuestas celulares (Figura 3; P&V) y humorales (Figuras 4 y 5: P&V) tras la infección demostró la persistencia de la respuesta Th1 hacia los antígenos vacunales correlacionada con la inducción de respuestas Th1 frente a los antígenos totales del parásito.

Estos resultados, junto a los comentados en el trabajo anterior de esta memoria, indican que las proteínas LmL3 y LmL5 podrían ser elementos relevantes para el desarrollo de vacunas definidas frente a infecciones con diferentes especies de *Leishmania*.

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Parasites & Vectors

RESEARCH Open Access

# Cross-protective effect of a combined L5 plus L3 *Leishmania major* ribosomal protein based vaccine combined with a Th1 adjuvant in murine cutaneous and visceral leishmaniasis

Laura Ramirez[1], Laura Corvo[1], Mariana C Duarte[2], Miguel A Chávez-Fumagalli[2], Diogo G Valadares[3], Diego M Santos[4], Camila I de Oliveira[4], Marta R Escutia[5], Carlos Alonso[1], Pedro Bonay[1], Carlos AP Tavares[3], Eduardo AF Coelho[2,6†] and Manuel Soto[1*†]

**Abstract**

**Background:** Two *Leishmania major* ribosomal proteins L3 (LmL3) and L5 (LmL5) have been described as protective molecules against cutaneous leishmaniasis due to infection with *L. major* and *Leishmania braziliensis* in BALB/c mice when immunized with a Th1 adjuvant (non-methylated CpG-oligodeoxynucleotides; CpG-ODN). In the present study we analyzed the cross-protective efficacy of an LmL3-LmL5-CpG ODN combined vaccine against infection with *Leishmania amazonensis* and *Leishmania chagasi (syn. Leishmania infantum)* the etiologic agents of different clinical forms of human leishmaniasis in South America.

**Methods:** The combined vaccine was administered subcutaneously to BALB/c mice. After immunization the cellular and humoral responses elicited were analyzed. Mice were independently challenged with *L. amazonensis* and *L. chagasi*. The size of the cutaneous lesions caused by the infection with the first species, the parasite loads and the immune response in both infection models were analyzed nine weeks after challenge.

**Results:** Mice vaccinated with the combined vaccine showed a Th1-like response against LmL3 and LmL5. Vaccinated mice were able to delay lesion development due to *L. amazonensis* infection and to control parasite loads in the site of infection. A reduction of the parasite burden in the lymph nodes draining the site of infection and in the liver and spleen was observed in the vaccinated mice after a subcutaneous infection with *L. chagasi*. In both models of infection, protection was correlated to parasite antigen-specific production of IFN-γ and down-regulation of parasite-mediated IL-4 and IL-10 responses.

**Conclusions:** The data presented here demonstrate the potential use of *L. major* L3 and L5 recombinant ribosomal proteins for the development of vaccines against various *Leishmania* species.

**Keywords:** *Leishmania* parasites, L3 and L5 ribosomal proteins, BALB/c mice, Experimental infection, Vaccines, Visceral leishmaniasis, Cutaneous leishmaniasis

* Correspondence: msoto@cbm.uam.es
†Equal contributors
[1]Centro de Biología Molecular Severo Ochoa (CSIC-UAM), Departamento de Biología Molecular. Nicolás Cabrera 1, Universidad Autónoma de Madrid, 28049 Madrid, Spain
Full list of author information is available at the end of the article

## Background

Infection with different species from the genus *Leishmania* can cause a variety of clinical symptoms known globally as leishmaniasis [1]. Although some veterinary vaccines against leishmaniasis are now available [2-4] no vaccine has been developed for humans. During the last few years some advances in the development of vaccines against leishmaniasis have been carried out [5]. Given that first generation vaccines using crude parasite antigens were unable to induce protection [6] two main strategies have been explored for the development of anti-*Leishmania* vaccines. The first one employ live vaccines composed of molecularly modified attenuated parasites (leishmanization) to induce protective anti-*Leishmania* immune responses [7,8]. Alternatively, second generation vaccines are based on the use of parasite protein fractions [9,10] or individual parasite antigens [11]. Although some of these second generation vaccines are currently used in human clinical trials [12] the screening of new candidates will help to further increase the prophylactic efficacy of a *Leishmania* vaccine. It has been proposed that combination of different parasite antigens may help to attain a vaccine containing the most appropriate protective characteristics [5]. In addition, since multiple *Leishmania* species are distributed in the same or adjacent geographical regions [13] it would be desirable to develop vaccines containing candidates capable of inducing protection against the infection caused by various *Leishmania* species. One example of this situation is South America where the leishmaniasis disease ranges from visceral forms (VL) caused by *Leishmania chagasi* (syn. *Leishmania infantum* [14]) infection, to cutaneous forms (CL) caused by infection with different parasite species such as *Leishmania braziliensis*, *Leishmania. pifanoi* and *Leishmania amazonensis*. All these species can coexist in different geographical regions [13]. Thus, to be effective as a human vaccine against leishmaniasis its components should be shared by different parasite species and, prior to its use in humans, the protective efficacy of these candidates should be analyzed in different models of experimental leishmaniasis.

Examples of such vaccine preparations are those based on parasite ribosomal proteins. It has been demonstrated that a preparation of biochemically purified *Leishmania* ribosomal proteins (LRP) administered in combination with Th1 inducing adjuvants conferred protection against the challenge with different parasite species: *L. amazonensis* and *L. chagasi* [15] or *L. major* promastigotes [16]. Also, vaccinated and protected BALB/c mice were able to control the disease due to a secondary parasite challenge [17]. It was recently reported that two of the large subunit constituents of *L. major* ribosomes L3 or L5, expressed as recombinant proteins (LmL3 and LmL5) and administered independently or in combination (always in the presence of a Th1 adjuvant such as non-methylated CpG-oligodeoxynucleotides; CpG-ODN) were able to control the outcome of infection in an experimental model of American CL, namely BALB/c mice infected with *L. braziliensis* [18]. Globally, it was found that protection was associated with both, the induction of LRP-, LmL3- or LmL5-specific IFN-γ mediated responses and the control of the antigen dependent production of the susceptibility associated cytokines IL-10 and IL-4 [19].

The first objective of the study was to analyze the immunogenic properties of a vaccine combining the LmL3 and LmL5 recombinant proteins and CpG-ODN as adjuvant in BALB/c mice. The second objective was to study the combined vaccine prophylactic properties, challenging immunized mice with two different *Leishmania* species: *L. amazonensis* and *L. chagasi*. The potential mechanism of the combined vaccine-induced observed protection was also investigated and is consistent with the maintenance of the Th1-like response against the LmL3 and LmL5 antigens induced by vaccination after infection.

## Methods

### Antigens and adjuvant

Soluble *Leishmania* antigenic (SLA) extract was prepared from stationary-phase promastigotes of *L. major*, *L. chagasi* and *L. amazonensis* as previously described [20]. *L. major* ribosomal proteins (LRP) or mouse ribosomal proteins (MRP) were prepared from logarithmic-phase promastigotes of *L. major* and RAW 264.7 murine macrophage cells, respectively, as previously described in [10].

LmL3 and LmL5 recombinant proteins were overexpressed in *Escherichia coli* (M15 strain), purified under denaturing conditions onto Ni-nitrilotriacetic-acid-agarose columns (Qiagen, Hilden, Germany) and refolded on the affinity column, as described in [21]. Polymyxin-agarose columns (Sigma, St. Louis. MO, USA) were employed to remove residual endotoxin content (<10 ng of LPS per 1 mg of recombinant protein, measured by the Quantitative Chromogenic Limulus Amebocyte Assay QCL-1000 (BioWhittaker, MD, USA)).

Phosphorothioate-modified CpG-ODN (5′-TCAACGT TGA-3′ and 5′- GCTAGACGTTAGCGT-3′) were synthesized by Isogen Life Science B.V. (De Meern, The Netherlands) and employed for their capacity to induce Th1 responses in mice when immunized with different leishmanial antigenic preparations [16,22].

### Immunization, challenge infection, cutaneous lesion development and parasite quantitation

The Bioethical Committee of the Consejo Superior de Investigaciones Científicas (CEEA-11/046) and the Universidad Autónoma de Madrid (CEI 21–443) in Spain and the Animal Use Committee of the Federal University of Minas

Gerais (CEUA; 047/2009) in Brazil approved the experimental. Mice (n = 4 or 5) were subcutaneously (s.c.) immunized in their left hind footpads with a mixture of the LmL3 and LmL5 recombinant proteins (6 μg each) plus 25 μg of each CpG-ODN (combined vaccine). As control groups, mice (n = 4 or 5) were inoculated with 25 μg of each CpG-ODN or with saline (PBS; vaccine diluent). Each group was boosted two and four weeks later with the same dose. For challenge, immunized mice were s.c. infected, into the right hind footpad, with $1 \times 10^7$ stationary-phase promastigotes of *L. chagasi* (n = 4, per group) or with $1 \times 10^6$ stationary-phase promastigotes of *L. amazonensis* (n = 4 per group). In mice infected with *L. amazonensis*, footpad swelling was measured with a metric caliper (the thickness of the left footpad minus thickness of the right footpad is shown). At week nine post-challenge all animals were sacrificed. For parasite load determination, the footpads of mice infected with *L. amazonensis* were taken and weighed before their individual processing. In addition the whole spleen, liver and the single popliteal lymph node draining the site of infection (DLN, right leg) of mice s.c. infected with *L. chagasi* were collected and independently processed as follows. Samples were mechanically homogenized in complete Schneider's medium (Schneider's medium (Sigma) supplemented with 20% heat-inactivated fetal bovine serum (FBS, Sigma), 20 mM L-glutamine, 200 U/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml gentamicin) and filtered using a cell strainer (70-μm pore size). Each homogenized sample tissue was serially diluted in a 96-well flat-bottomed microtiter plate containing the same medium (in triplicates). The number of viable parasites (by mg of tissue for the footpads and by organ in the spleen, liver and DLN) was determined from the highest dilution at which promastigotes could be grown with up to 10 days of incubation at 25°C as previously described [23].

### Mice and parasites

Female BALB/c mice (6–8 weeks old) were purchased from Harlan (BCN, Spain) or from the Institute of Biological Sciences, ICB, Federal University of Minas Gerais (Belo Horizonte, Brazil). First, the immunization procedure was carried out using a total number of 15 mice (5 mice immunized with saline, 5 mice immunized with the adjuvant and 5 mice immunized with the combined vaccine). Mice were euthanized one month after the last immunization for the analysis of the immune response elicited by vaccination. Next, mice were immunized subcutaneously with the combined vaccine (n = 12), with the vaccine diluent (n = 12) or with the vaccine adjuvant (n = 12). One month after vaccination, 4 mice per group were euthanized to test the reproducibility of the vaccine induced response. The remaining animals were infected s.c. with *L. amazonensis* (n = 4 mice per group) or *L. chagasi* (n = 4 per group) to analyze the effect of vaccination in leishmaniasis progression. This last assay was reproduced using the same number of mice.

Regarding parasites, *L. major* clone V1 (MHOM/IL/80/Friedlin), *L. chagasi* (MOM/BR/1970/BH46) and *L. amazonensis* (IFLA/BR/1967/PH-8) parasites were grown at 25°C in complete Schneider's medium.

### Cytokine production

Spleen cells obtained from each mouse were seeded and independently cultured in RPMI complete medium at $5 \times 10^6$ cells per ml (RPMI medium (Sigma) supplemented with 10% heat-inactivated FBS, 20 mM L-glutamine, 200 U/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml gentamicin) during 48 h at 37°C in 5% $CO_2$ alone or with some of the next stimuli: recombinant LmL3 (12 μg/ml), recombinant LmL5 (12 μg/ml), SLA (from the indicated species, 12 μg/ml), LmLRP (12 μg/ml) and MRP (12 μg/ml). The release of IFN-γ, IL-10 and IL-4 was measured in culture supernatants by sandwich ELISA using monoclonal antibodies specific for mouse cytokines (capture and detection) provided in commercial kits (Pharmingen, San Diego, CA, USA), following the manufacturer's instructions.

### Analysis of the humoral responses

Animals (n = 4 per group) were bled four weeks after the last immunization and nine weeks after challenge and the anti-LmL3-, anti-LmL5- or anti-SLA specific IgG1 and IgG2a antibodies present in the sera were measured by ELISA, as described elsewhere [20]. Briefly, 96-well plates (Becton Dickinson, Franklin Lakes, NJ, USA) were sensitized with the recombinant proteins or SLA (from the indicated species) at 10 μg/ml (each one) in PBS (100 μl/well) for 16 h at 4°C. Plates were blocked with PBS-10% bovine serum albumin at 37°C for 1 h and treated with 1/200 dilutions of mouse serum samples for 2 h at 37°C. Peroxidase-conjugated anti-mouse IgG1 or IgG2a isotype (Sigma) was diluted at 1:5,000 (for recombinant proteins) or 1:10,000 (for SLA) and added for 1 h at 37°C. Reactions were developed by incubation with $H_2O_2$ and O-phenylenediamine. Optical densities were read at 492 nanometers in a spectrophotometer (Molecular Devices, Spectra Max Plus, Concord, Canada).

### Statistical analysis

Statistical analysis with the vaccinated and infected mice was performed by a two-tailed Student's *t*-test. Differences were considered significant when $P < 0.05$.

## Results and discussion

### Immunogenicity of the LmL3 + LmL5 + CpG-ODN combined vaccine in BALB/c mice

Since combination of different parasite protective antigens have been defined as an adequate strategy for *Leishmania* vaccine development [24,25] we decided to

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test a LmL3 + LmL5 + CpG-ODN combined vaccine based on the observation that both proteins were able to induce protection against murine CL due to *L. major* infection [19]. Moreover, administration of the CpG-ODN adjuvant combined with these antigens as single or combined vaccines induced a robust protection in mice against infection with a mixture of *L. braziliensis* stationary-phase promastigotes and insect vector saliva, while no protection was observed when animals were only treated with CpG-ODN [19]. However, the immune response elicited against the antigens by their co-administration was not analyzed in that work.

From an immunological point of view, we show that spleen cells from mice immunized with the combined vaccine were able to secrete LmL3- and LmL5-specific IFN-γ, since significantly higher levels of this cytokine were found in the culture supernatants after stimulation with these antigens, when compared with the culture supernatants established from control mice, namely saline ($P = 0.0006$ for LmL3 and $P = 0.0002$ for LmL5) and CpG-ODN groups ($P = 0.0002$ for both antigens) (Figure 1A). The presence of the CpG-ODN, an agonist of the TLR9 [26] that confers a Th1-related long-term immunity and protection when combined with different leishmanial antigens [22] was essential for the stimulation of the antigen specific IFN-γ response, since low levels of this cytokine were produced when the LmL3 + LmL5 proteins were co-administered in the absence of the adjuvant (not shown).

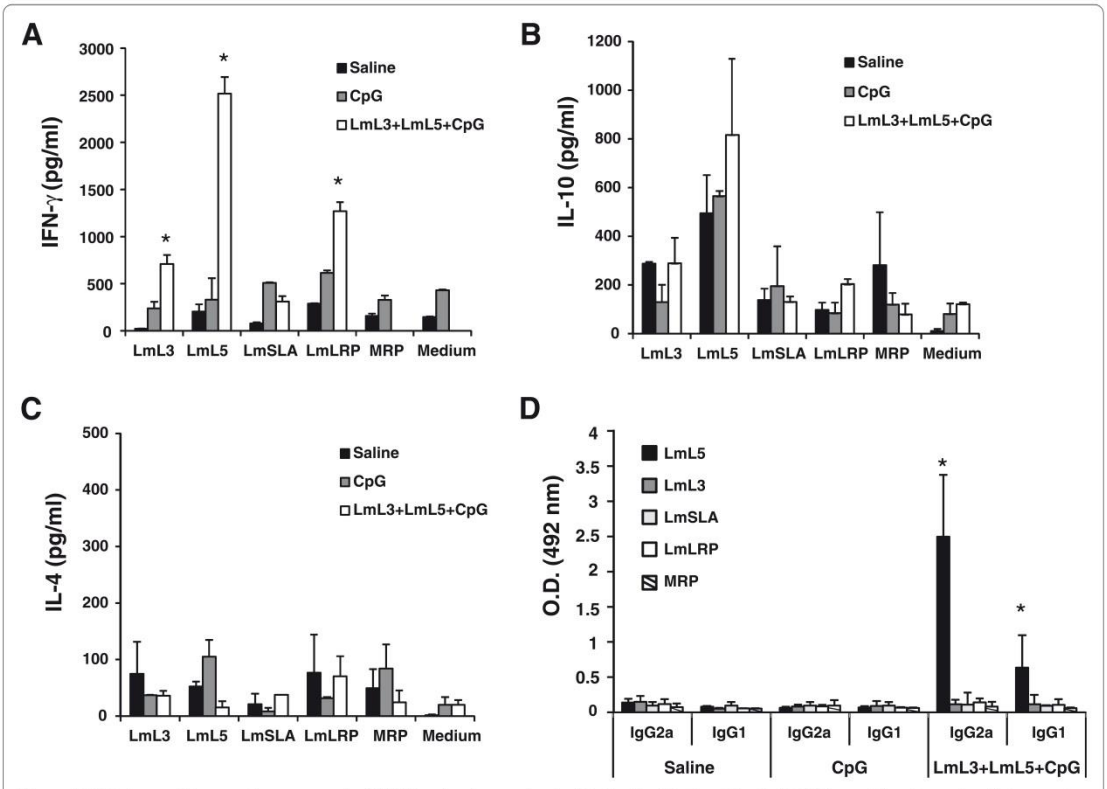


**Figure 1 Cellular and humoral responses in BALB/c mice immunized with the LmL3 + LmL5 + CpG-ODN combined vaccine.** Spleen cell cultures were established four weeks after the last vaccine dose and maintained for 48 h at 37°C, 5% $CO_2$. As control, spleen cell cultures from mice immunized with the vaccine diluent (Saline) and the CpG-ODN adjuvant (CpG) were prepared at the same time. Splenocytes were grown without stimulus (medium; background control) or independently stimulated with the recombinant LmL3 or LmL5 proteins (at 12 μg/ml each one), with Soluble *L. major* Antigen (LmSLA), *L. major* Ribosomal Proteins (LmLRP) or Mouse Ribosomal Proteins (MRP) (at 12 μg/ml for each extract). **(A)** IFN-γ, **(B)** IL-10 and **(C)** IL-4 levels were assessed by ELISA in culture supernatants. Each bar represents the mean + standard deviation (SD) of data from five individual mice. * $P < 0.05$; significant cytokine production increase between LmL3 + LmL5 + CpG-ODN vaccinated mice and both control groups. **(D)** IgG1 and IgG2a antibodies against LmL3, LmL5, SLA, LRP and MRP were measured in serum samples from the three mice groups. Samples were obtained four weeks after the last vaccine dose and individually tested by ELISA at 1/200 dilution. Each bar represents the mean + SD of data from five individual mice. * $P < 0.05$; significant increase in the optical density between LmL3 + LmL5 + CpG-ODN vaccinated mice and both control groups. Data shown are representative of at least two independent experiments with similar results.

The IFN-γ mediated response was comparable to that induced when each one of the antigens was administered in the presence of the same adjuvant [19]. The LmL3 and LmL5 combined vaccine also induced similar patterns of IL-10 and IL-4 secretion than single based vaccines [19]. Spleen cells from vaccinated mice showed a slightly higher production of IL-10 than control mice groups when stimulated with LmL5, although the differences were not significant (Figure 1B). Very low similar levels of IL-4 were detected in vaccinated and control groups after stimulation with both recombinant proteins (Figure 1C). In addition, vaccinated mice showed a specific anti-LmL5 humoral response that was predominantly of the IgG2a isotype ($P = 0.01$ compared with saline and CpG-ODN groups) although IgG1 antibodies specific for this antigen were also observed in the sera from immunized mice ($P = 0.03$ compared with saline and CpG-ODN groups) (Figure 1D). It can be concluded that the vaccine combining LmL3, LmL5 and CpG-ODN induced a predominant Th1-like response for both antigens of different magnitude, stronger for LmL5 than for LmL3, because of the robust production of LmL5-specific IFN-γ and by the presence of anti-LmL5 IgG2a antibodies, markers of Th1-type responses [27]. The immune response elicited against the recombinant proteins was correlated with a production of IFN-γ when *L. major* ribosomes (LmLRP) were employed for *in vitro* stimulation (Figure 1A; LmLRP). The lack of LmSLA-specific production of cytokines (Figure 1A-C; LmSLA) may be related to the lower contents of ribosomal proteins in a total parasite extract when compared to the LmLRP preparation.

*Leishmania* LmL3 and LmL5 proteins were selected for a cross-protection analysis due to their high degree of conservation among different *Leishmania* species [19]. On the other hand, and regarding host counterparts, *L. major* LmL3 and LmL5 showed lower identity and similarity scores with respect to their mouse homologs: *Mus musculus* L3 (NCBI: NP_038790.2), identity: 59.4%, similarity: 74.0%; *Mus musculus* L5 (NCBI: NP_000960.2), identity: 53.4%, similarity: 68.5%. Remarkably, no humoral or cellular responses against host ribosomal proteins were observed in vaccinated mice (Figure 1A-D; MRP). This lack of immune cross-reactivity between parasite and host intracellular proteins belonging to conserved families was also observed for other intracellular antigens, such as histones and heat shock proteins in infected individuals [28]. This observation has been related to the location of B and T cell epitopes in the most divergent regions of these parasite proteins [28-30].

#### Effects of vaccination in the development of CL due to *L. amazonensis* and VL due to *L. chagasi* in BALB/c mice

Since the development of vaccines against leishmaniasis requires the definition of potential candidates capable of inducing protective responses against different *Leishmania* species [12], we evaluated the protective efficacy of the combined vaccine (LmL3 + LmL5 + CpG-ODN) in two different forms of murine leishmaniasis. The first one was a model of infection with a high dose of *L. amazonensis* stationary promastigotes in the footpad of BALB/c mice. This highly pathogenic species is able to cause different forms of American Tegumentary leishmaniasis (ATL) and VL in humans and a severe CL in mice [31,32]. Mice immunized with the combined vaccine showed a reduction in the lesion size when compared with control mice groups immunized with saline or with the adjuvant alone (Figure 2A). Differences were significant until nine weeks after infection when compared with saline and until seven weeks when compared with CpG-ODN group (Figure 2A). At the end of the assay (nine weeks after challenge), the parasite burdens found in the infected footpads were lower in vaccinated mice than in both controls groups (Figure 2B).

The decrease in parasite loads in the infected footpads was statistically significant when compared with both groups ($P = 1.6 \times 10^{-5}$ and $P = 4.5 \times 10^{-5}$ for saline and CpG-ODN groups, respectively). It was concluded that although the administration of the adjuvant alone could have a slight influence on the outcome of infection, the administration of the combined vaccine induced a delay of the progressive disease due to the *L. amazonensis* challenge. The BALB/c-*L. amazonensis* model of infection has been employed for a limited number of antigenic preparations including some antigens described as protective in other forms of the disease like the LACK protein or the amastigote A2 antigen (reviewed in [11]) and the *Leishmania* P4 nuclease, a protein immunogenic in humans infected with *L. major* [33] and related to protection against experimental murine *L. pifanoi* infection [34]. Among them, the most protective formulation was based on defined antigens administered in combination with IL-12: P4 nuclease [35] or the A2 protein [20], since no footpad swelling was observed after *L. amazonensis* challenge. Although it is difficult to establish a direct comparison between different vaccine candidates (due to the use of different parasite strains, number of parasite in the inoculum, adjuvants employed, etc.), the combined vaccine assayed here seems to be inducing weaker protection than vaccines that employ IL-12 as adjuvant, a cytokine that plays a central role in promoting Th1 responses and cell-mediated immunity [36]. The effects of the combined vaccine are more comparable with other vaccines based on parasite total proteins [37-39], antigenic extracts [38-40] including LRP + saponin [15] and some DNA vaccines based on the A2 [41] or LACK [39] proteins, that induced a delay in the footpad swelling. Of interest, the 4.5-log reduction observed in the footpad parasite loads of mice vaccinated with the LmL3 + LmL5 + CpG-ODN

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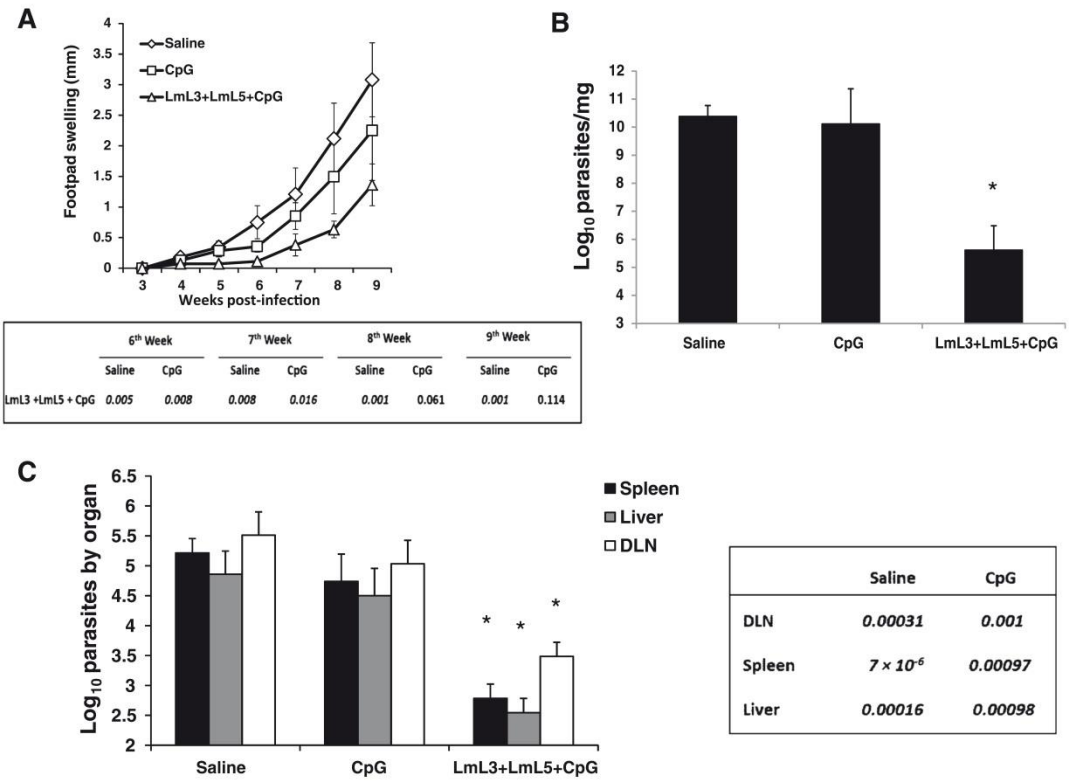


| | 6th Week | | 7th Week | | 8th Week | | 9th Week | |
|---|---|---|---|---|---|---|---|---|
| | Saline | CpG | Saline | CpG | Saline | CpG | Saline | CpG |
| LmL3 +LmL5 + CpG | *0.005* | *0.008* | *0.008* | *0.016* | *0.001* | 0.061 | *0.001* | 0.114 |

| | Saline | CpG |
|---|---|---|
| DLN | *0.00031* | *0.001* |
| Spleen | *$7 \times 10^{-6}$* | *0.00097* |
| Liver | *0.00016* | *0.00098* |

**Figure 2 Effect of the vaccination in murine leishmaniasis due to *L. amazonensis* or *L. chagasi* infection.** Mice (four per group) immunized with vaccine diluent (Saline) with CpG-ODN adjuvant (CpG) or with the recombinant vaccine (LmL3 + LmL5 + CpG) were independently challenged subcutaneously in the left footpad with $1 \times 10^6$ stationary-phase promastigotes of *L. amazonensis* **(A-B)** or $1 \times 10^7$ stationary-phase promastigotes of *L. chagasi* **(C)**. **(A)** Lesion development was monitored weekly during nine weeks in mice infected with *L. amazonensis*. The mean ± standard deviation (SD) of the footpad swelling is given as the difference of thickness between the infected and the uninfected contralateral footpad. *P* values (saline immunized versus vaccinated or CpG-ODN immunized versus vaccinated) are included in the table below the graph. **(B)** Parasite burden was determined in the infected footpads (parasites per mg) nine weeks after infection with *L. amazonensis*. Mean + SD is shown. * $P < 0.05$ indicates significant decrease in the footpad parasite burdens between vaccinated and both control groups. **(C)** Parasite burdens (parasites per organ) in the spleen, in the liver and in the popliteal lymph node draining the infected footpad (DLN) after *L. chagasi* infection. Mean + SD of four mice in each group is shown. *P* values (saline immunized versus vaccinated or CpG-ODN immunized versus vaccinated) are included in the table at the right of the graph. Data shown are representative of two independent experiments with similar results.

preparation with respect to both control groups (Figure 2B) was comparable in magnitude with parasite burden differences observed in mice vaccinated with the most protective formulations and their controls [20,35,41]. Moreover, it should be taken into account that some antigen-based vaccines, such as LACK combined with IL-12 [20] or a DNA vaccine based on a Nucleoside Hidrolase [41] an antigen that induces partial protection against other *Leishmania* species [34], were unable to control murine CL due to *L. amazonensis* infection. In addition, some parasite serine-proteases were able to exacerbate the *L. amazonensis* related disease when immunized as a prophylactic preparation alone or combined with saponin [42].

A great number of different molecules have been tested as second generation vaccines in murine models of VL infection [43]. Most of the tested antigens were studied using the intravenous route of infection that guarantee the induction of VL but could undervalue the potential efficacy of some vaccines [44]. We decided to subcutaneously challenge *L. chagasi* in the footpad of BALB/c mice because this model has been accepted as an optimal screening tool to analyze protective antigens [45] and has been previously employed to test the immunoprophylactic properties of different parasite components [46,47], including the combination of LRP and saponin [15]. As it is shown in Figure 2C the *L. chagasi* challenge resulted in parasite active infection with the presence of

parasites in the DLN (in the absence of footpad swelling) but also in the spleen and in the liver, internal organs involved in parasite replication in murine VL [48]. Nine weeks after infection, mice immunized with LmL3 + LmL5 + CpG-ODN showed significantly lower parasite burdens than both control groups in the three analyzed organ locations (Figure 2C). The decrease of parasite loads in the vaccinated mice was more evident in the internal organs (2.5-log reduction in the liver and in the spleen when compared with saline group and 2-log reduction in the liver and in the spleen when compared with the CpG-ODN group). A significant decrease in the parasite burdens of the lymph node draining the infected footpad (2-log and 1.5-log reduction when compared with saline and CpG-ODN groups, respectively) was also observed. As it also occurred after *L. amazonensis* challenge (the present study) and other cutaneous species (*L. major* and *L. braziliensis* [19]) vaccinated mice were able to control the replication of different *Leishmania* species, allowing the conclusion that LmL3 and LmL5 based vaccines will fit the requirements to a pan-*Leishmania* vaccine.

#### Immunological parameters associated with protection

To determine the immunological parameters of protection, the production of different cytokines in the supernatants of spleen cell cultures, established from the different groups of mice and stimulated with different antigenic preparations was analyzed.

First, the post-infection response against the antigens composing the vaccine was examined. Vaccinated and infected mice showed an IFN-γ LmL3- and LmL5-specific production that was absent in the infected control groups. A significant increase in the level of this cytokine in the supernatant of vaccinated versus saline and CpG spleen cells cultures was observed after stimulation with LmL3: $P = 0.0006$ for both antigens in *L. amazonensis* infected mice (Figure 3A) and $P = 0.0008$ and $P = 0.0007$, respectively, in *L. chagasi* infected mice (Figure 3B). Similarly, stimulation with LmL5 resulted in the secretion of higher levels of IFN-γ in vaccinated than in saline and CpG control mice groups: $P = 0.0008$ for both antigens in *L. amazonensis* infected mice (Figure 3A) and $P = 2 \times 10^{-5}$ and $P = 3 \times 10^{-5}$, respectively, in *L. chagasi* infected mice (Figure 3B). We did not detect antigen specific IL-4 or IL-10 production after stimulation with the recombinant LmL3 or LmL5 proteins in the vaccinated and infected mice. Thus, it was concluded that after challenge the Th1-like response elicited against the LmL3 and LmL5 ribosomal proteins by the vaccine was maintained. Following infection, cellular responses elicited against LmL5 were higher than those detected against LmL3. The different immunogenic properties of the two recombinant proteins were also observed in the humoral responses elicited against both proteins in the infected mice. In both mouse models, *L. amazonensis* vaccinated and infected mice (Figure 4A) or *L. chagasi* vaccinated and infected mice (Figure 4B) the reactivity against LmL5 was stronger than against LmL3, as also occurred in mice vaccinated with these proteins after *L. major* infection [19]. In the *L. amazonensis* model higher IgG2a anti-LmL3 and anti-LmL5 antibody reactivity were observed in vaccinated than in saline mice groups ($P = 0.0063$ and $P = 0.0006$, respectively) or in CpG-ODN immunized mice ($P = 0.002$ and $P = 0.001$, respectively) (Figure 4A). After *L. chagasi* infection only the IgG2a anti-LmL5 antibodies showed higher reactivity in vaccinated than in control mice ($P = 0.002$ saline and $P = 0.003$ CpG-ODN) (Figure 4B).

The systemic cellular response in infected BALB/c mice against *L. amazonensis* parasite total proteins was analyzed by LaSLA (prepared from this parasite specie) stimulation of spleen cell cultures established from saline and CpG-ODN control groups. SLA specifically induced the secretion of IL-4 and IL-10 production in the absence of IFN-γ production in both infected control groups (Figure 3A). This low T- cell response also reported for other experimental infections performed with *L. amazonensis* [15,20,41,49] can negatively affect the activation of the effector functions of macrophages for destroying the intracellular amastigotes [31,50]. On the contrary, after infection vaccinated mice mounted a LaSLA-specific IFN-γ mediated response (vaccinated mice versus saline [$P = 0.0005$] or CpG-ODN [$P = 0.0006$] groups) (Figure 3A), that may be activating the macrophages for destruction of parasites at the infection site, resulting in the observed decrease of the parasite load shown in Figure 2B. As reported here, induction of parasite specific IFN-γ responses by vaccination was also related to protection induced by parasite lysates [15,38,39] or single parasite proteins based vaccines [35,39]. In addition, a decrease in the LaSLA-mediated IL-4 and IL-10 responses was observed in the vaccinated and infected mice (Figure 3A). A comparison between saline and CpG-ODN controls and protected mice revealed that spleen cells from vaccinated mice produced significantly lower amounts of IL-4 ($P = 2 \times 10^{-5}$ and $P = 0.0002$, respectively) and IL-10 ($P = 0.002$ and $P = 0.0001$, respectively). A decrease in IL-10 was correlated with protection induced by the P4 Hidrolase protein + IL-12 vaccine [35] and a decrease of IL-4 and IL-10 parasite-specific mediated responses was also observed in mice protected by the administration of LRP + saponin [15], although the exact role of these cytokines in the development of lesions due to *L. amazonensis* is not fully understood [31]. The humoral responses elicited against total parasite proteins were in accordance with the

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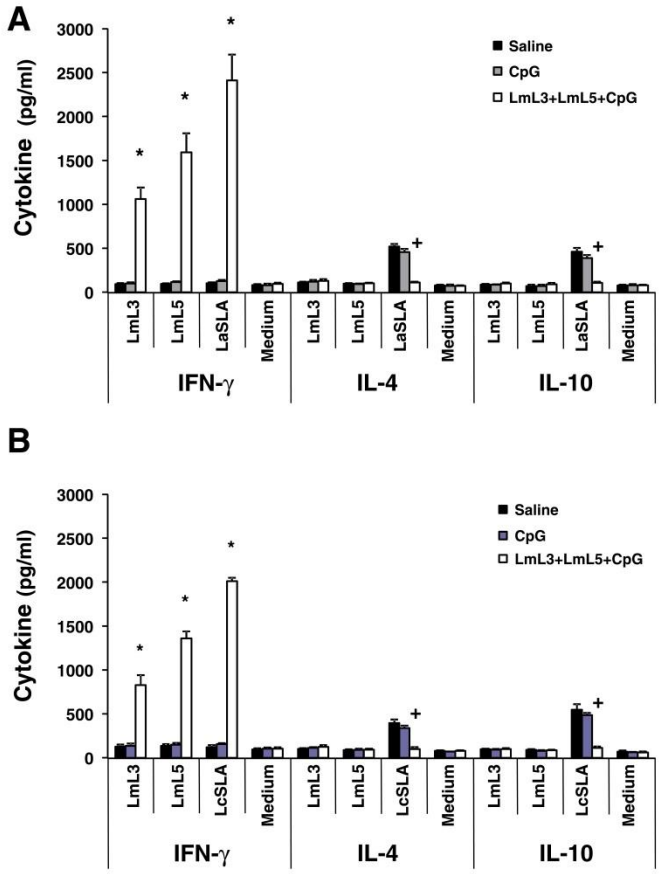


**Figure 3 Production of cytokines by spleen cells of vaccinated mice after *Leishmania* infection.** Mice (four per group) immunized with vaccine diluent (Saline), with CpG-ODN adjuvant (CpG) or with the recombinant vaccine (LmL3 + LmL5 + CpG) were independently challenged subcutaneously in the left footpad with $1 \times 10^6$ stationary- phase promastigotes of *L. amazonensis* **(A)** or $1 \times 10^7$ stationary-phase promastigotes of *L. chagasi* **(B)**. Nine weeks after infection, spleen cell cultured suspensions were non-stimulated (Medium; background control) or separately stimulated with recombinant LmL3 or LmL5 (12 μg/ml each one) or with SLA from *L. amazonensis* (LaSLA; A) or *L. chagasi* (LcSLA, B) at 12 μg/ml each one for 48 h at 37°C, 5% $CO_2$. IFN-γ , IL-4 and IL-10 levels were measured in culture supernatants by ELISA. Mean + standard deviation (SD) of cytokine levels determined in four individual mice per group is shown; *, $P < 0.05$ indicates significant cytokine production increase between LmL3 + LmL5 + CpG-ODN vaccinated mice and both control groups; +, $P < 0.05$ indicates significant cytokine production decrease between LmL3 + LmL5 + CpG-ODN vaccinated mice and both control groups. Data shown are representative of two independent experiments with similar results.

cellular responses induced by infection, since anti-LaSLA antibodies found in the vaccinated mice were mainly of the IgG2a isotype, being the anti-LaSLA reactivity of the IgG1 isotype antibodies significantly lower than those detected in both control groups ($P = 0.0006$ versus saline and $P = 0.0007$ versus CpG-ODN, respectively) (Figure 5).

Similarly, when the cellular responses elicited in BALB/c mice infected with *L. chagasi* against the LcSLA (using extracts prepared from this parasite specie) were analyzed, a LcSLA-specific production of IFN-γ was detected in the vaccinated mice that was absent in saline ($P = 5 \times 10^{-9}$) or CpG-ODN ($P = 4 \times 10^{-7}$) groups (Figure 3B). This response was correlated to the predominant presence of anti-LcSLA IgG2a antibodies in the sera from vaccinated and infected mice (Figure 5). Since IFN-γ dependent activation of infected macrophages for production of nitric oxide is necessary for *Leishmania* intracellular killing [51] this cytokine has been considered one of the main factors implicated in the acquired immunity against infection with viscerotropic *Leishmania* species [15,41,52,53]. IL-10

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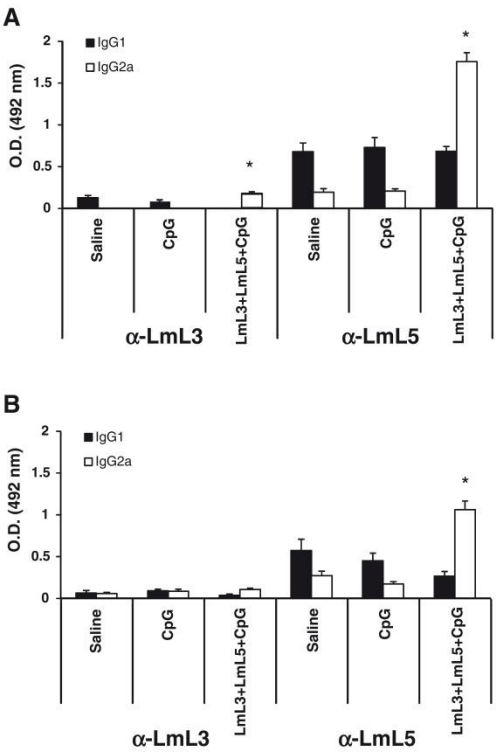


**Figure 4 Humoral response against the recombinant antigens following infection with *L. amazonensis* (A) or *L. chagasi* (B).** Nine weeks after parasite challenge vaccinated and control mice (n = 4 per group) were bled and sera were individually tested by ELISA for specific anti-LmL3 and anti-LmL5 antibody responses of both IgG1 and IgG2a isotype at 1/200 dilution. Each bar represents the mean + standard deviation (SD) of data from four individual mice. * $P < 0.05$ indicates significant increase in the optical density between LmL3 + LmL5 + CpG-ODN vaccinated mice and both control groups. Data shown are representative of two independent experiments with similar results.

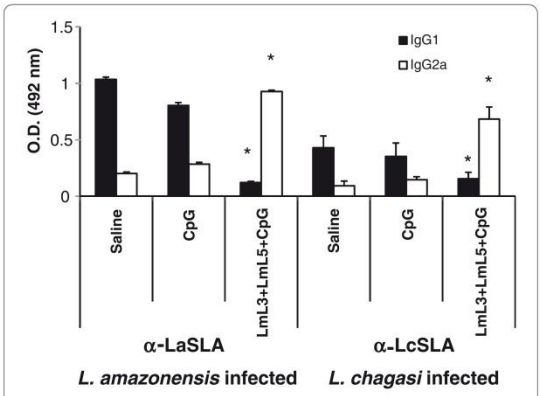


**Figure 5 Humoral response against soluble parasite antigens (SLA) antigens following infection with *L. amazonensis* or *L. chagasi*.** Nine weeks after parasite challenge vaccinated and control mice (n = 4 per group) were bled and sera were individually tested by ELISA for specific anti-SLA antibody responses of both IgG1 and IgG2a isotypes at 1/200 dilution. Each bar represents the mean + standard deviation (SD) of data from four individual mice. * $P < 0.05$ indicates significant changes in the optical density between LmL3 + LmL5 + CpG-ODN vaccinated mice and both control groups. Data shown are representative of two independent experiments with similar results.

parasite mediated responses are critical for VL progression [54], since BALB/c mice lacking the gene for IL-10 [55] or BALB/c mice treated with an anti-IL-10 receptor antibody [56] are resistant to infection. In accordance, mice vaccinated with the LmL3 + LmL5 + CpG-ODN combined vaccines showed a specific decrease in the LcSLA-mediated IL-10 ($P = 0.0004$ for saline and $P = 8 \times 10^{-7}$ for CpG-ODN) (Figure 3B) and also a controlled production of IL-4 specific for the parasite antigens ($P = 7 \times 10^{-5}$ for saline and $P = 2 \times 10^{-5}$ for CpG-ODN) (Figure 3B) correlated to the presence of low levels of anti-LcSLA IgG1 reacting antibodies (Figure 5). Although the implication of IL-4 mediated responses in murine VL progression has not been clearly demonstrated, various reports have correlated the induction of protection against a subcutaneous challenge with *L. chagasi* to the control of *Leishmania*-specific IL-4 mediated responses [15,41,46,47].

## Conclusions

The present study indicated that the administration of a vaccine based on the combination of the recombinant versions of the *L. major* ribosomal proteins L3 and L5 in the presence of a Th1 adjuvant (CpG-ODN) conferred cross-protection in BALB/c mice against subcutaneous infection with two different *Leishmania* species: *L. amazonensis* and *L. chagasi*. After vaccination, mice showed an LmL3, LmL5 and LRP (*Leishmania* ribosomal proteins) Th1-like response as shown by the production of IFN-γ specific for these antigens, in the absence of IL-10 or IL-4-specific responses. In spite of the conserved nature of the ribosomal proteins, vaccinated mice did not show cellular and humoral responses against the ribosomal protein of the vertebrate host (MRP extract). The immune response against LmL3 and LmL5 elicited by the combined vaccine was maintained after infection in the vaccinated and protected mice. Protection was also correlated with the induction of parasite dependent IFN-γ responses and with the down-regulation of parasite dependent IL-4 and IL-10 responses. Since LmL3 and LmL5 based vaccines were able to induce protection against different *Leishmania* species in BALB/c mice (*L. amazonensis* and *L. chagasi*; this work) and other cutaneous species such as *L. major* and *L. braziliensis* [19] we may conclude that these antigens could play a relevant role as components of a pan-*Leishmania* vaccine.

**Competing interests**
The authors declare that they have no competing interests.

**Authors' contributions**
LR, LC, MCD, MACF, DGV, MRE and MS carried out the experimental procedures. MACF, DMS, CIO, MRE, CA, PB, CAPT, EAFC and MS conceived the research, contributed with data analysis and revised the manuscript. EAFC and MS wrote the manuscript. All authors read and approved the final version of the manuscript.

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**Author details**
[1]Centro de Biología Molecular Severo Ochoa (CSIC-UAM), Departamento de Biología Molecular. Nicolás Cabrera 1, Universidad Autónoma de Madrid, 28049 Madrid, Spain. [2]Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical, Faculdade de Medicina, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil. [3]Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil. [4]Centro de Pesquisas Gonçalo Moniz, FIOCRUZ, Salvador, Bahia, Brazil. [5]Research & Development Department, Laboratorios LETI S.L.u, 28760 Madrid, Spain. [6]Departamento de Patologia Clínica, COLTEC, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil.

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# DISCUSIÓN


讨论
Discussion
مناقشة
Обсуждение
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อภิปราย
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Eztabaida

El desarrollo de vacunas contra las diferentes formas de leishmaniosis humanas es, en la actualidad, una línea de investigación en desarrollo. La posibilidad de diseñar vacunas se basa en la observación de que los pacientes recuperados de la enfermedad son resistentes a la reinfección con la misma especie y que incluso en algunos casos son capaces de generar protección cruzada frente a otras especies (Porrozzi, Teva et al. 2004). Por otra parte, la aparición de tres vacunas comerciales frente a la leishmaniosis canina apoya esta hipótesis: Leishmune® (basada en extractos extracelulares del parásito combinados con saponina) (Dantas-Torres 2006), Leishtech® (basada en una versión recombinante de la proteína A2 específica del amastigote) (Fernandes, Coelho et al. 2012) y Canileish (compuesta de factores secretados/excretados del promastigote y una fracción purificada de la saponina) (Moreno, Vouldoukis et al. 2012; Bongiorno, Paparcone et al. 2013; Moreno, Vouldoukis et al. 2014). Estas vacunas comerciales han de ser consideradas como un primer paso hacia la completa profilaxis de la leishmaniosis canina ya que ninguna de ellas es capaz de inducir una protección del 100% (Fernandes, Coelho et al. 2012; Otranto and Dantas-Torres 2013). Las tres formulaciones pueden ser clasificadas dentro de las vacunas de segunda generación; aquellas basadas en fracciones o en antígenos individuales del parásito (Palatnik-de-Sousa 2008). La combinación de los antígenos con adyuvantes adecuados (normalmente inductores de respuestas celulares (Ferdousi, Alam et al. 2012)) es fundamental para la obtención de una respuesta protectora. En su origen, los antígenos que componen las vacunas fueron probados en modelos de leishmaniosis experimental en el ratón (Santos, Paraguai de Souza et al. 1999; Santarem, Silvestre et al. 2007; Zanin, Coelho et al. 2007) antes de ser ensayados en las especies receptoras finales. Estos datos parecen validar las investigaciones realizadas para la caracterización de antígenos vacunales empleando modelos experimentales, aunque éstos no reflejen en su totalidad la evolución clínica y parasitológica de los pacientes con infecciones naturales, como ocurre en los modelos de LC y LV en el ratón. Estos modelos son muy empleados en la caracterización de moléculas para el desarrollo de vacunas contra las leishmaniosis humanas. Existen diferentes candidatos, ensayados en diversos modelos de leishmaniosis en el ratón y en otros modelos de laboratorio como hámster y macaco (Soto, Ramírez et al. 2009; Nylen and Gautam 2010; Mutiso, Macharia et al. 2013), entre los que destaca una proteína quimérica denominada Leish-111f (= LEISH-F1). Esta proteína recombinante está formada por la fusión de tres antígenos del parásito; TSA, LmSTI1 y LeIF, muy estudiados en distintos

modelos experimentales en los que han generado respuestas protectoras frente a diferentes especies de *Leishmania* (Alvar, Croft et al. 2013). Posteriormente fue evaluada como potencial vacuna contra la leishmaniosis humana en diferentes ensayos de seguridad e inmunogenicidad (fase 1) (Alvar, Croft et al. 2013; Beaumier, Gillespie et al. 2013) combinada con MPL (monofosforil lípido A de *Salmonella minnesota*) un adyuvante permitido para su uso en humanos (Reed, Orr et al. 2013). Los prometedores resultados condujeron a la elaboración de una versión mejorada, Leish-110f (LEISH-F2) sin fusiones para la purificación y con la eliminación de una secuencia de proteolisis (Bertholet, Goto et al. 2009), que se ha empleado para estudios en fase 1 y fase 2 (eficacia) en el tratamiento de pacientes de LC (Beaumier, Gillespie et al. 2013). Otra proteína de fusión compuesta de dos antígenos probados de forma independiente como protectores en el ratón, (la nucleósido hidrolasa (Al-Wabel, Tonui et al. 2007) y la 24-c-metiltransferasa (Goto, Bogatzki et al. 2007; Goto, Bhatia et al. 2009)), está siendo analizada en un ensayo de fase 1 (Beaumier, Gillespie et al. 2013).

En este trabajo se ha realizado una investigación dirigida al empleo de las proteínas ribosómicas del parásito como vacunas contra la leishmaniosis. El empleo de esta partícula como fuente de antígeno se basó en resultados publicados con anterioridad al comienzo de esta Tesis Doctoral, y se ha ido reforzando con los resultados aquí presentados y los publicados por otros autores durante el tiempo de realización de esta investigación. Como se discutirá en este apartado, los ribosomas (o sus componentes) tienen diferentes características que los hacen interesantes para el desarrollo de vacunas al cumplir las condiciones de lo que se considera una vacuna ideal frente a la leishmaniosis (Alvar, Croft et al. 2013):

- Contienen moléculas que interaccionan con el sistema inmunológico en diferentes formas de leishmaniosis.
- Son inmunogénicos, y cuando se combinan con adyuvantes adecuados generan respuestas celulares.
- Están conservados en las diferentes especies del parásito y están presentes en las formas promastigotes y amastigotes.
- Son capaces de generar inmunidad frente a diferentes especies del parásito.

- La respuesta dirigida hacia ellos es específica de los componentes ribosómicos del parásito, requisito indispensable que debe ser analizado en detalle por la naturaleza conservada de éstos.

La antigenicidad de las proteínas del ribosoma queda perfectamente demostrada en los resultados mostrados en la primera referencia de esta Tesis Doctoral (CVI). Entre los componentes proteicos del ribosoma se localizan un gran número de proteínas antigénicas reconocidas por un elevado número de perros infectados de forma natural por *L. infantum*, independientemente de la sintomatología clínica que presentaban (Figuras 1 y 2; CVI). La naturaleza antigénica de las proteínas del mismo ya se había demostrado para algunos de los componentes proteicos del ribosoma, como las proteínas ácidas en la LV canina (Soto, Requena et al. 1995; Soto, Requena et al. 1995; Soto, Requena et al. 1996). En este trabajo se ha evidenciado la antigenicidad de las proteínas de la subunidad mayor del ribosoma L3 y L5 y de la subunidad pequeña S4 y S6 (Figura 1: V). La antigenicidad no es específica de la especie del parásito, ni está asociada exclusivamente a la LV, forma de la enfermedad que genera una fuerte respuesta humoral en humanos (Herwaldt 1999) y perros (Baneth, Koutinas et al. 2008), ya que se ha comprobado que las proteínas antes indicadas son también antigénicas en pacientes con LMC causada por la infección por *L. braziliensis* (Figura 1; V). De hecho, y por datos obtenidos en la referencia 1 (CVI), los ribosomas podrían ser considerados una interesante alternativa al SLA para el diagnóstico diferencial de la infección por *Leishmania* y la infección por *T. cruzi* o *Toxoplasma gondii,* ya que combina un similar grado de sensibilidad y una mejor especificidad (Figuras 2 y 3; CVI). La mejora en el diagnóstico se produce, sobre todo, en la determinación temprana de la leishmaniosis, ya que un elevado porcentaje de animales asintomáticos reconocen la fracción LRP. Esta detección temprana, podría facilitar un pronto tratamiento, mejorando así la eficacia terapeútica (Ferdousi, Alam et al. 2012).

La generación de anticuerpos no es la única evidencia que ha relacionado la respuesta inmunológica frente a los ribosomas con la progresión de la enfermedad. Así, y empleando modelos experimentales, se comprobó que la proteína S3a de *L. major* es capaz de generar una reducción de las respuestas Th1 e inducción de respuestas de linfocitos B policlonales (Cordeiro-Da-Silva, Borges et al. 2001), la inmunización de la proteína L31 produce una exacerbación de la enfermedad induciendo respuestas mediadas por IL-4 e IL-10 (Roberts, Stober et al. 2005) y la inmunización con la proteína ácida ribosómica P2a induce respuestas Th2 en ratones, aunque sea

administrada con adyuvantes inductores de respuestas Th1 (Iborra, Abanades et al. 2007). Finalmente, y regresando a la partícula completa, se ha demostrado que los ratones BALB/c infectados por *L. major* presentan respuestas celulares y humorales mayoritariamente asociadas a la progresión de la patología (producción de respuestas mediadas por IL-4 e IL-10 frente a las proteínas ribosómicas del parásito, así como la inducción de respuestas de anticuerpos de la subclase IgG1 contra un gran número de ellas) (Iborra, Parody et al. 2008). Sin embargo, los datos existentes indican que la interacción del sistema inmunológico con el ribosoma del parásito puede resultar, en ocasiones, en la inducción de respuestas asociadas con resistencia. La primera evidencia de este tipo fue la producción de respuestas Th1 frente a la proteína S4 en pacientes resistentes a la infección por *L. chagasi* y pacientes recuperados de la patología causada por la infección con *Leishmania tropica* (Probst, Stromberg et al. 2001). Esta observación en pacientes se corrobora con la demostración de que la inmunización con proteínas recombinantes o vacunas de DNA basadas en algunas proteínas ribosómicas (P1, P0, S19 y L22) (Iborra, Soto et al. 2003; Iborra, Carrion et al. 2005; Stober, Lange et al. 2006; Bhardwaj, Vasishta et al. 2009; Masih, Arora et al. 2011) o de combinaciones de plásmidos codificantes para varias proteínas ribosómicas (Melby, Ogden et al. 2000) genera diferentes grados de protección en animales de experimentación. Finalmente, y como evidencia experimental importante para el desarrollo del trabajo aquí presentado, resultados previos del laboratorio demostraron que las fracciones LRP de *L. major* eran capaces de proteger a los ratones frente a la infección por *L. major* (Iborra, Parody et al. 2008). Esta protección sólo ocurre cuando la fracción antigénica ribosómica es administrada en presencia de un adyuvante (CpG-ODN) capaz de generar respuestas celulares mediadas por linfocitos Th1, que ya había demostrado ser útil para la inducción de respuestas protectoras frente a *Leishmania* (Rhee, Mendez et al. 2002) y para el control de las respuestas asociadas con la patología (Zimmermann, Egeter et al. 1998; Chiaramonte, Hesse et al. 2000). La hipótesis de partida que se planteó era que si la vacunación es capaz de redirigir la respuesta Th2 generada por los ribosomas durante el proceso infectivo hacia una respuesta de tipo Th1 se podría conseguir controlar la patología (Campos-Neto 2005; Soto, Ramírez et al. 2009). En el trabajo de Iborra y col. (Iborra, Parody et al. 2008) la vacunación de las LRP junto con CpG-ODN generó protección tanto en ratones susceptibles como resistentes y se correlacionó con la generación de respuestas mediadas por IFN-γ en

ambos modelos y con el control de las respuestas asociadas a patología (mediadas por IL-4 e IL-10) en el modelo susceptible.

En la segunda referencia de esta Tesis Doctoral (JBB) se continuó con la evaluación de la protección inducida por la misma vacuna en ratones susceptibles (BALB/c) infectados por *L. major* por periodos de tiempo más largos. Tras la infección, se monitorizó la aparición de síntomas clínicos en el sitio de infección (almohadilla plantar) a lo largo de dieciocho semanas (alrededor del 20% de la vida de un ratón BALB/c hembra (Festing and Blackmore 1971)). Durante este tiempo (bastante prolongado para la rápida evolución de la patología en este modelo) no se observaron lesiones. Los animales presentaron una inmunidad protectora (respuestas LRP o SLA específicas mediadas por IFN-γ en ausencia de secreción de IL-4 e IL-10) sin observarse la dispersión de parásitos hacia el bazo típica de este modelo (BALB/c-*L. major*) (Handman 2001). Este estado de protección se acompañaba con la presencia de parásitos en el ganglio poplíteo que drena la zona de la infección. Se concluyó que la vacuna de ribosomas generaba una infección crónica asintomática y controlada en el lugar de infección. La cronificación de la infección se ha considerado fundamental para el mantenimiento de la inmunidad ya que si los ratones eliminan por completo el parásito, no se genera una memoria eficiente para resistir infecciones posteriores (Uzonna, Wei et al. 2001; Okwor and Uzonna 2008; Okwor, Mou et al. 2012). Teniendo en cuenta que los hospedadores vertebrados residentes en regiones endémicas pueden ser infectados más de una vez, se evaluó la evolución clínica y parasitológica en los ratones protegidos tras una segunda infección. Cuando estos animales fueron reinfectados (en este caso en la oreja, y con otra cepa de *L. major*) apenas aparecieron lesiones. La ausencia de lesiones puede deberse a la rápida respuesta celular producida por la vacunación y mantenida gracias a la infección crónica del ganglio poplíteo. Esta rápida respuesta, se ve reflejada en la baja carga parasitaria encontrada en los ganglios retromandibulares, las orejas y el bazo de los animales reinfectados. Es importante destacar que incluso en los ratones resistentes a la infección por *Leishmania*, (por ejemplo los C57BL/6) aparecen lesiones inflamatorias durante las primeras etapas de la infección hasta que el hospedador es capaz de controlar la infección (Belkaid, Kamhawi et al. 1998). En los ratones reinfectados se detectó la reactivación de la infección (un incremento de más de un millón de parásitos en el ganglio poplíteo, siete semanas después de la segunda infección) que se puede explicar en base a datos publicados previamente que demuestran que las reinfecciones pueden provocar una reactivación de

los parásitos contenidos en el primer sitio de infección. Esto se debe a la migración de las células efectoras desde el lugar de la primera infección al sitio de la nueva infección, lo que resulta en una posible disminución en el control del crecimiento de los parásitos en el sitio primario (Mendez, Reckling et al. 2004). El análisis de las respuestas inmunológicas post-infección en los animales empleados en este ensayo (controles y reinfectados), demostró que las células de los ganglios retromandibulares estimuladas con LRP producen más IFN-γ en los animales controles que en los reinfectados (Figura 3; JBB). Este dato puede ser debido a la mayor carga parasitaria de los controles, ya que se conoce que en este modelo la producción de esta citoquina se correlaciona con la cantidad de parásitos (Courret, Lang et al. 2003). Sin embargo, en los cultivos de esplenocitos se produce el efecto contrario, siendo los ratones protegidos los que producen una mayor cantidad de IFN-γ. Este hecho (concordante con la existencia de anticuerpos del isotipo IgG2a específicos de los antígenos del parásito (Figura 4; JBB)) refuerza la idea de la existencia de una respuesta Th1 sistémica, asociada a la resistencia a la reinfección. Además, los animales protegidos tras la segunda infección no mostraron niveles apreciables de IL-4 e IL-10, contrariamente a los animales que presentaban lesiones. Se puede concluir que la vacunación con LRP en presencia del adyuvante CpG-ODN es una formulación adecuada para el desarrollo de vacunas contra la leishmaniosis causada por la infección por *L. major*, ya que protege tanto a ratones susceptibles como a resistentes frente a una infección primaria (Iborra, Parody et al. 2008), generando en los animales susceptibles un estado de resistencia. Este estado de resistencia es similar al conseguido por la leishmanización (administración controlada de dosis subclínicas de parásitos infecciosos (Courret, Lang et al. 2003)) o la administración de parásitos atenuados (Beattie, Evans et al. 2008; Makala, Baban et al. 2011). Al igual que en el caso de los animales protegidos vacunados con LRP y CpG-ODN, la leishmanización produce el mantenimiento de una infección crónica subclínica que provoca una respuesta de células T efectoras asociada con la resistencia (Uzonna, Wei et al. 2001; Belkaid, Piccirillo et al. 2002). Esto ocurriría de forma natural en los pacientes vacunados que, residiendo o visitando zonas endémicas para el patógeno quedarían leishmanizados en ausencia de clínica. Se cumple entonces una de las condiciones que requiere un candidato para crear vacunas contra *Leishmania*, la inducción de respuestas celulares protectoras capaces de controlar la infección con el patógeno (Handman 2001; Alvar, Croft et al. 2013). En este aspecto, quedarían por

realizar ensayos de duración de inmunidad para poder determinar el periodo en el que la vacuna generaría un estado de protección ante la primera infección con el parásito.

Un inconveniente de las vacunas basadas en una purificación bioquímica de los ribosomas puede ser la estandarización de la preparación de diferentes lotes de vacuna y la problemática asociada al desarrollo de un proceso a gran escala, ya que se requiere el crecimiento de células (promastigotes en cultivo), así como la purificación de componentes por ultracentrifugación. Buscando solucionar esta problemática, se decidió estudiar individualmente la capacidad protectora de algunos de los componentes proteicos del ribosoma para poder aplicar técnicas recombinantes en el desarrollo de las vacunas. Para ello, se aislaron los genes codificantes de las proteínas de mayor tamaño del ribosoma al ser ésta una zona que contiene proteínas reconocidas por un porcentaje elevado de sueros de perros con LVC (Figura 1) y ratones infectados por *L. major* (Iborra, Parody et al. 2008). Se empleó la base de datos del genoma de *L. major* como genoteca genómica *in silico* para localizar las secuencias génicas codificantes por las proteínas ribosómicas. Esta estrategia permitió obtener cuatro proteínas recombinantes equivalentes a las proteínas ribosómicas denominadas LmL3, LmL5, LmS6 y LmS4. La estrategia de clonaje y expresión se explica en la cuarta referencia de esta Tesis Doctoral y se ve facilitada por la ausencia de intrones en el genoma de estos parásitos (Clayton 2002). Básicamente, se realiza la amplificación por PCR de las regiones codificantes para las cuatro proteínas, empleando como molde DNA genómico de los promastigotes. Las secuencias obtenidas se clonan en vectores de expresión de *E. coli* y las proteínas se purifican, tras su sobre-expresión, por cromatografía de afinidad en columnas de níquel-agarosa (por la interacción con una fusión de seis histidinas en su extremo amino-terminal). Las cuatro proteínas resultaron ser antigénicas en LV caninas y humanas provocadas por la infección con *L. infantum* y *L. chagasi*, respectivamente, y en pacientes afectados de LMC provocada por la infección con *L. braziliensis* (Figura 1; V). Siguiendo el mismo diseño desarrollado para LRP (Iborra, Parody et al. 2008), se valoró su capacidad de controlar la infección con *L. major* en ratones BALB/c tras su administración individual en presencia o en ausencia de adyuvante (CpG-ODN). De las ocho formulaciones probadas (además de las formulaciones control basadas en el excipiente o el adyuvante), sólo dos fueron capaces de generar inmunidad frente a la infección: LmL3 + CpG-ODN y LmL5+ CpG-ODN (Figura 2; V). Ambas proteínas eran inmunogénicas (en un mayor grado la proteína LmL5) y generaban respuesta Th1 en los ratones cuando se administraban en presencia del adyuvante (Figura 3; V). La

protección observada (basada en la evolución más lenta de las lesiones en las almohadillas plantares y en la presencia de menor número de parásitos en los ganglios poplíteos y en el bazo que los animales controles) era comparable, aunque en un grado menor, a la observada en el mismo modelo con la fracción LRP. Este dato sugería que la mezcla de antígenos que supone la inmunización con LRP podría generar respuestas más completas que las generadas por la inmunización de los antígenos individuales, como se ha descrito para otras preparaciones vacunales del parásito. Un ejemplo son las vacunas basadas en las cuatro histonas nucleosómicas del parásito que administradas en combinación, generan protección frente a diferentes especies de *Leishmania* (Iborra, Soto et al. 2004; Carrion, Folgueira et al. 2008; Carneiro, Santos et al. 2012) disminuyendo esta protección cuando se administran individualmente (Carrion, Folgueira et al. 2008). Cuando se analiza la respuesta inmunológica tras la infección contra las proteínas vacunales y las proteínas totales del ribosoma (Figuras 4 y 5; V) se observa que la inmunización de LmL5 + CpG-ODN es capaz de inducir una mayor producción de IFN-γ en los animales infectados, mientras que la formulación LmL3 + CpG-ODN parece inducir protección controlando las respuestas asociadas a la producción de IL-10. Según este resultado, una combinación de ambos antígenos podría ser capaz de generar un mayor grado de protección al combinar los dos efectos deseados: inducción de respuestas Th1 y control de las respuestas mediadas por IL-10; efecto observado en los animales vacunados con LRP + CpG-ODN. Esta hipótesis se retomará posteriormente cuando se analice el efecto protector de los antígenos definidos en la infección por otras especies del parásito. Se concluye este párrafo con la demostración de que los ribosomas de *L. major* (y algunos de sus componentes) son capaces de inducir protección frente a la infección con este patógeno y que las vacunas pueden llegar a generarse con proteínas definidas del ribosoma, cumpliéndose así otro de los requisitos de una vacuna contra *Leishmania*.

El siguiente punto de análisis fue la comprobación de la capacidad de los ribosomas de inducir protección en modelos de infección con otras especies del parásito diferentes a *L. major*. En el primer ensayo (que se presenta aquí como la referencia número tres; M&I) se probó la capacidad profiláctica de vacunas preparadas con los ribosomas de *L. infantum* (LRP) en la infección por *L. chagasi* (genéticamente idéntica a *L. infantum* (Mauricio, Stothard et al. 2000)) y *L. amazonensis* (especie empleada para comprobar la protección cruzada). Las LRP se combinaron en este caso con saponina, adyuvante validado para su uso veterinario que, como se indicó anteriormente, es la

base de la respuesta generada por la vacunas comerciales frente a la LV canina y que se está evaluando como adyuvante en vacunas para humanos (Sun, Xie et al. 2009). A continuación, y en una segunda fase de esta parte de este proyecto de Tesis, se validó la protección generada por las proteínas LmL3 y LmL5 en estos dos modelos (referencia cinco, P&V) así como en la infección en el ratón por *L. braziliensis* (referencia cuatro; V).

La respuesta inducida en los ratones BALB/c por la combinación de LRP y saponina presentó un perfil muy similar al obtenido con el adyuvante genético, al inducirse respuestas mediadas por IFN-γ (IL-12 dependientes) y anticuerpos contra LRP mayoritariamente de la subclase IgG2a (Figura 1; M&I). Los ratones fueron infectados con *L. chagasi* de forma subcutánea con una dosis elevada de promastigotes estacionarios, reto capaz de generar una infección visceral (Oliveira, Costa et al. 2012). Los animales vacunados presentaban menor carga parasitaria en el hígado (donde los parásitos se multiplican en las primeras etapas de la infección y luego son eliminados, al activarse por IFN-γ los macrófagos infectados (Carrion, Nieto et al. 2006)) y en el bazo (donde los parásitos cronifican (Wilson, Jeronimo et al. 2005; Oliveira, Costa et al. 2012)). Precisamente en este órgano, y en los ratones vacunados, se observó una respuesta mediada por IFN-γ, y lo que es destacable, se redujo la producción específica de IL-10 (Figura 4; M&I), punto importante al ser esta linfoquina la responsable de la falta de control en este modelo (Stager, Joshi et al. 2010) e incluso en humanos que padecen LV (Nylen, Maurya et al. 2007). De forma similar, la vacunación con LRP combinada con saponina tiene un efecto en la LC causada por la infección con *L. amazonensis*. En este modelo se observó un descenso de la carga parasitaria en la almohadilla plantar y en el bazo de los ratones vacunados con respecto a los ratones control. Sin embargo, en los ratones vacunados se observó el desarrollo de lesiones progresivas en la almohadilla plantar infectada. Éstas, eran menores que en los controles y retrasaron su aparición en el tiempo en los ratones vacunados (Figura 3; M&I). Este retraso se correlacionó con una mayor inmunidad Th1 en los animales vacunados respecto a los controles (mayor producción de IFN-γ y menores respuestas mediadas por IL-10 e IL-4 [Figura 5; V]). El estado inmunológico generado parece no ser suficiente para el completo control de la patología en este modelo de ratón, donde se ha descrito que *L. amazonensis* es más patogénica que *L. major* (Pereira and Alves 2008). Tampoco se puede descartar que las diferencias se deban al empleo de un adjuvante diferente, saponina en el modelo de *L. amazonensis* y CpG-ODN en el modelo *L. major*. Se

concluye que las vacunas basadas en los ribosomas cumplen otro requisito para ser tomados en consideración para el desarrollo de vacunas, ya que son capaces de generar protección frente a diferentes especies, y además, la protección puede ser cruzada, ya que los ribosomas de *L. infantum* inducen protección frente a la infección por *L. amazonensis.*

La protección cruzada es un aspecto enormemente importante, ya que se ha descrito que la inmunización con antígenos de una especie del parásito puede condicionar la infección con otras especies. Así, como ejemplo, la administración de proteínas totales de *L. amazonensis* puede aumentar la infectividad de *L. braziliensis* en modelos de ratón (Silva, Larangeira et al. 2011; de Araujo, Silva et al. 2014). De la misma forma, la infección por algunas especies en humanos o en animales de experimentación (monos y ratones) puede no generar una inmunidad protectora (revisado en (Porrozzi, Teva et al. 2004)). La protección cruzada ha de estar basada en un elevado grado de conservación de los antígenos vacunales entre las especies de los parásitos y en la presunción de que los antígenos de elección generen respuestas inmunológicas similares en las diferentes formas de infección (revisado en (de Oliveira, Nascimento et al. 2009)). Con los antígenos definidos sí se ha podido establecer el grado de conservación de las proteínas ribosómicas en diferentes especies del género *Leishmania*. Como ejemplo, y empleando los datos contenidos en las bases de datos de los genomas de *Leishmania*, se ha detectado un elevado grado de conservación para algunas proteínas entre dos especies del patógeno (Figura suplementaria 2B; V). Estos estudios se han ampliado, demostrándose un elevado grado de conservación de diversas proteínas ribosómicas entre diferentes especies del parásito (dato no mostrado). Existen evidencias de protección cruzada tras el empleo de vacunas basadas en proteínas recombinantes del ribosoma, destacando los realizados con la proteína ácida P0, localizada en la subunidad mayor del ribosoma. En concreto, la proteína P0 de *L. infantum* induce protección frente al reto con *L. major* en ratones BALB/c y C57BL/6 mediante la generación de respuestas celulares (Iborra, Soto et al. 2003; Iborra, Carrion et al. 2005). Por otra parte, la generación de respuestas humorales hacia la proteína P0 de *Neospora caninum* se ha postulado como una posibilidad de protección frente a la neosporosis y frente a la toxoplasmosis al presentar los anticuerpos generados una reactividad cruzada para la proteína P0 de *T. gondii*, (Zhang, Lee et al. 2007) proteína ribosómica que se ha localizado en la superficie de diferentes protozoos del filo apicomplexa (Singh, Sehgal et al. 2002; Sehgal, Kumar et al. 2003).

En primer lugar, se estudió el efecto de las vacunas basadas en LmL3 y LmL5 en el modelo de infección BALB/c-*L. braziliensis.* Este modelo fue descrito por primera vez en la referencia (de Moura, Novais et al. 2005) y consiste en la inoculación intradérmica de una alta dosis de promastigotes estacionarios de *L. braziliensis*. Se caracteriza por la generación de una lesión inflamatoria (mediada fundamentalmente por linfocitos Th1) en la oreja infectada que provoca la destrucción del parásito. Para aumentar la virulencia del agente infectante se añadió en el inóculo infectivo una mezcla de proteínas de las glándulas salivares del insecto vector, ya que se ha descrito que estos factores facilitan la infección (Samuelson, Lerner et al. 1991; de Moura, Oliveira et al. 2007). La vacunación se realizó administrando las proteínas de forma individual o combinada, siempre en presencia de CpG-ODN. En relación a la respuesta inmunológica asociada a la co-inoculación de ambas proteínas (Figura 1; P&V), se puede resumir que resulta en una respuesta similar a la suma de las respuestas observadas en las vacunaciones individuales (Figura 3; V); una respuesta Th1 hacia ambos antígenos, de mayor intensidad para la proteína LmL5. Las tres formulaciones indujeron un elevado grado de protección caracterizado por la ausencia de lesiones inflamatorias en las orejas infectadas y una patente disminución de la carga parasitaria en el lugar de infección (Figura 6; V). Esta disminución de los parásitos en la dermis de la oreja no se apreciaba en el ganglio retromandibular, donde se observaban cargas parasitarias similares en los controles y los vacunados. Esta presencia se correlaciona con el mantenimiento de una respuesta Th1 en los ganglios linfáticos infectados. Este hecho sugiere que el estado inmunológico generado por la vacuna fue capaz de alterar la proliferación de los parásitos en las orejas, y por lo tanto, el tamaño de las lesiones inflamatorias, aunque los parásitos se mantienen en el ganglio en una infección crónica. La protección observada en este modelo es de especial relevancia, ya que muchas de las vacunas experimentales ensayadas con éxito en otros modelos no fueron capaces de generar protección frente a la patología causada por *L. braziliesis,* incluyendo aquellas basadas en los antígenos que conforman las vacunas experimentales LEISH-F1 y LEISH-F2 (Salay, Dorta et al. 2007) mencionadas en el primer párrafo de esta discusión. De forma similar, los antígenos excretados por *L. major* son capaces de generar inmunidad en los ratones BALB/c frente a la infección por *L. donovani*, pero no son capaces de controlar la infección por *L. braziliensis* (Tonui and Titus 2007). Se continuó la evaluación de la capacidad protectora de la vacuna de combinación LmL3 + LmL5 + CpG-ODN en los modelos ya empleados con LRP: BALB/c infectados por *L.*

*chagasi* y por *L. amazonensis* (referencia número cinco; P&V). En ambos modelos la infección se vió alterada como resultado de la vacunación. Globalmente, y tras analizar la carga parasitaria, se detectó un efecto protector en ambos modelos, apreciándose una disminución significativa del número de parásitos en los lugares de infección analizados en los animales vacunados en relación a los controles del ensayo (animales inmunizados con el tampón salino donde se formuló la vacuna o con el adyuvante empleado) (Figura 2 BC; P&V). No obstante, se apreció una evolución progresiva de las lesiones en las almohadillas plantares de los animales vacunados, más lenta que en los controles (Figura 2 A; P&V). La protección, como en casos anteriores, se correlacionó con el mantenimiento de las respuestas mediadas por IFN-γ contra los antígenos vacunales generados por la vacunación, y la generación de IFN-γ junto al control de la secreción de IL-10 e IL-4 frente a SLA (Figura 3; P&V). Todos estos resultados permiten afirmar que las vacunas basadas en proteínas ribosómicas, así como las que están formuladas a partir de LmL3 y LmL5, redundan en un estado inmunitario que promueve un interesante grado de protección en los modelos en ratón de diferentes leishmaniosis humanas.

El empleo de ribosomas como partículas capaces de producir protección frente a *Leishmania* requiere un cuidadoso análisis de la especificidad de la respuesta generada por estas partículas. *Leishmania* es un eucariota y las proteínas ribosómicas, al igual que otras familias proteicas, son moléculas pertenecientes a familias conservadas. Los ribosomas no son las únicas proteínas conservadas que son reconocidas por el sistema inmunológico del hospedador vertebrado tras la infección. Las histonas, las proteínas de choque térmico y algunos los componentes del complejo de iniciación de la traducción son también antigénicos en infecciones naturales (revisado en (Requena, Alonso et al. 2000)). Es importante destacar que en la mayor parte de los trabajos donde se ha analizado la especificidad de la respuesta frente a estas proteínas, los resultados han demostrado que los pacientes (incluso aquellos que presentaban fuertes respuestas humorales) reconocen de forma específica las proteínas del parásito sin presentar reactividad cruzada frente a los ortólogos del hospedador (Requena, Alonso et al. 2000). La base de esta especificidad se encuentra en que las proteínas conservadas del parásito presentan regiones divergentes en las que se localizan los epítopos B (Quijada, Requena et al. 1996; Soto, Requena et al. 1999; Carmelo, Zurita et al. 2006; Santarem, Silvestre et al. 2007) y los epítopos T (Probst, Stromberg et al. 2001; Antoine, Prina et al. 2004). Este importante aspecto se ha tenido en cuenta en los estudios realizados sobre el papel

profiláctico de los ribosomas de *Leishmania*. Iborra y colaboradores demostraron que en el suero de ratones BALB/c vacunados con los extractos ribosómicos e infectados por *L. major*, no se detectaban anticuerpos frente a las proteínas ribosómicas del ratón (Iborra, Parody et al. 2008). En relación a la inmunización de LmL3 y LmL5 administradas junto a CpG-ODN, tanto de forma independiente como en combinación, los resultados mostrados en la Figura 3; V y la Figura 1; P&V indican que las células del bazo de los animales vacunados no sintetizan citoquinas tras la estimulación con extractos ribosómicos preparados de células de ratón. Estas evidencias parecen apoyar la especificidad de la respuesta producida hacia las proteínas ribosómicas, aunque este importante aspecto deberá analizarse no sólo en modelos de animales sanos, sino también en modelos que pueden producir respuestas autoinmunes.

Para concluir esta discusión, una reflexión sobre los ribosomas como antígenos. El empleo de vacunas basadas en los ribosomas de diferentes patógenos ha sido una práctica común en el pasado para inducir inmunidad frente a infecciones, sobre todo bacterianas, pero también generadas por hongos, helmintos y protozoos (Gregory 1986; Gao, Wang et al. 2010). La inmunidad en muchos casos se asocia con la generación de anticuerpos frente a las proteínas del ribosoma, los cuales son capaces de interferir en la infección o dirigir el sistema inmune hacia los patógenos (Pregliasco, Terracciano et al. 2009). La presencia de proteínas del ribosoma en la superficie de bacterias (Severin, Nickbarg et al. 2007) y protozoos (Singh, Sehgal et al. 2002; Sehgal, Kumar et al. 2003) explica la inmunidad humoral, aunque no se ha descartado que en las fracciones ribosómicas empleadas para preparar las vacunas se puedan encontrar proteínas de localización en la superficie celular (como péptidos nacientes asociados a los ribosomas polisómicos o como contaminaciones). En otros casos, se ha descrito una inmunidad celular asociada con la vacunación con las proteínas del ribosoma (Eckstein, Barenholz et al. 1997; Pregliasco, Terracciano et al. 2009), especialmente en infecciones con patógenos intracelulares como *Brucella*, *Mycobacterium* y *Babesia* (Gregory 1986; Terkawi, Jia et al. 2007). Estas respuestas protectoras se han conseguido no sólo con las fracciones ribosómicas, sino también con versiones recombinantes de proteínas ribosómicas (LmL3 y LmL5 en este trabajo; V y P&V) y otros componentes ribosómicos ((Iborra, Soto et al. 2003; Baloglu, Boyle et al. 2005; Iborra, Carrion et al. 2005; Stober, Lange et al. 2006; Zhang, Lee et al. 2007; Gao, Wang et al. 2010)). Esta última observación, aunque no descarta la implicación de factores no ribosómicos asociados con la partícula del ribosoma, pone de manifiesto que parte de las proteínas

estructurales del ribosoma son capaces de interaccionar con el sistema inmunológico del hospedador vertebrado para generar respuestas mediadas por linfocitos B. La localización extracelular de algunos componentes ribosómicos (en la superficie celular o en los productos excretados (Severin, Nickbarg et al. 2007; Jain, Kumar et al. 2014)) podría explicar esta relación. Según los datos presentados en la primera referencia de esta Tesis Doctoral (Figura 1; CVI) y en trabajos previos del laboratorio (Iborra, Parody et al. 2008) muchos de los componentes proteicos del ribosoma son antigénicos tras la infección con *Leishmania*. La interacción de estas proteínas con los linfocitos B puede ocurrir por liberación de los ribosomas tras la lisis de los parásitos mediada por el complemento (Mosser and Edelson 1984; Ambrosio and De Messias-Reason 2005), o por acción de las NETs (Guimaraes-Costa, Nascimento et al. 2009). La presencia de diferentes proteínas ribosómicas en las fracciones secretadas/excretadas por el parásito puede ser una forma alternativa de presentación de estos antígenos al sistema inmunológico del hospedador (Silverman, Chan et al. 2008).

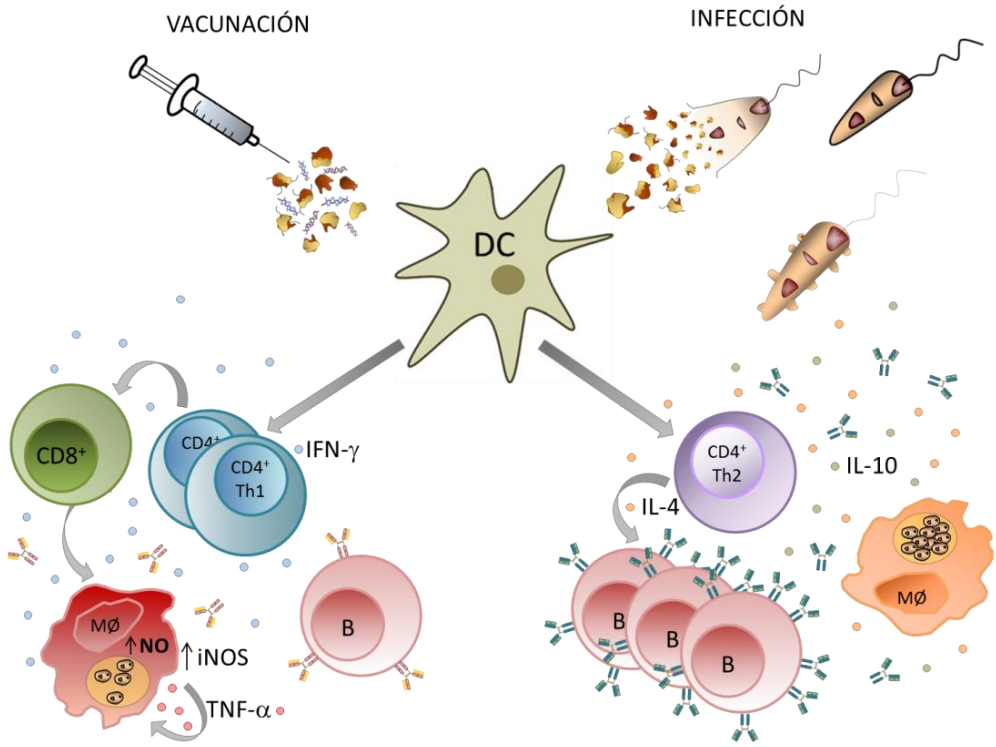


**Figura 4: Esquema de la respuesta inmunológica producida por la vacunación y por la infección.**

Tras la presentación, se generarían respuestas humorales frente a las proteínas ribosómicas (por ejemplo, y entre otras, hacia la L5) así como respuestas mediadas por

IL-10 (como en el caso de L3). Estas respuestas favorecerían la progresión de la infección. La vacunación con las proteínas del ribosoma en presencia de adyuvantes estimuladores de respuestas celulares generan una memoria celular del tipo Th1 que, en el momento de la infección y tras reconocer proteínas del ribosoma del parásito, es capaz de contrarrestar las respuesta natural hacia estos antígenos. El control de la infección provocaría una infección crónica en ausencia de síntomas clínicos capaz de mantener la inmunidad para contrarrestar sucesivas rondas infectivas.

# CONCLUSIONES

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1. Los ribosomas son partículas inmunodominantes en la infección con diferentes especies de *Leishmania*. La generación de anticuerpos es independiente de la sintomatología y muestran mayor especificidad que el reconocimiento de SLA.
2. Los ratones vacunados con LRP + CpG-ODN e infectados con *L. major* son capaces de resistir a una segunda infección. Mantienen una elevada respuesta celular de tipo Th1 y una disminución de las respuestas mediadas por IL-10 gracias al mantenimiento de una respuesta T efectora producida por la cronificación de los parásitos en el ganglio drenante de la infección primaria.
3. La vacunación con LRP + Saponina es capaz de producir la protección frente a la infección por diferentes especies de *Leishmania* (*L. chagasi* y *L. amazonensis*). En los animales vacunados hay una reducción de la carga parasitaria en los órganos diana de la infección acompañada de un retraso en la aparición de lesiones cutáneas de los animales infectados por *L. amazonensis.*
4. Las proteínas L3 y L5 del ribosoma de *L. major* son antigénicas durante infecciones naturales tanto en humanos como en perros. Las correspondientes versiones recombinantes (LmL3 y LmL5) son capaces de producir respuestas inmunes tras su inoculación en ratones BALB/c.
5. La inmunización de las proteínas LmL3 y LmL5 combinadas con el adjuvante CpG-ODN genera un estado inmunitario capaz de controlar la infección por *L. major* en ratones susceptibles BALB/c. La protección se relaciona con el control de las respuestas mediadas por IL-10 en el caso de la proteína LmL3 y la inducción de elevados niveles de IFN-γ en el caso de LmL5.
6. La vacuna compuesta por la combinación de LmL3, LmL5 y CpG-ODN es capaz de generar una protección cruzada frente a la infección con diferentes especies de *Leishmania* (*L. chagasi*, *L. braziliensis* y *L. amazonensis*) en ratones BALB/c.
7. Los ribosomas (o alguno de sus componentes proteicos) han de ser considerados serios candidatos para la elaboración de una vacuna eficaz frente a los diferentes tipos de leishmaniosis.

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